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PREFACE

This introduction to the twelfth volume of the *Review* is written in a spirit of optimism that has been denied to us except on rare occasions throughout the turbulent years of the past decade. Perhaps our optimism rests in the hope that we entertain for an early end to the devastating struggle in which the entire world has been engulfed and in the hope that the preservation of freedom in the postwar world will be demanded with a universal insistence to which there can be no denial. Some of us perchance, on this thirteenth day of April, have found a new courage in those cardinal principles enunciated by Thomas Jefferson—principles of government to which all who seek to be free would gladly subscribe.

Lest it be deemed by those who read these pages that such matters are far removed from the recognized concerns of those who prepare and edit scientific papers we need but remind ourselves of several extraordinary impasses to which we have been brought in recent years, and which bear very directly upon our immediate responsibilities. The interchange of information in all fields of knowledge proceeds with difficulty and by devious channels between the two parts of the world. The publication of work in the sciences is hampered by numerous restrictions the necessity of which in time of war can hardly be gainsaid. Censorship, even between the nations that are allies in a common cause, imposes unusual problems. The printing, sale, and transportation of publications in all fields of learning no longer proceed according to the usual conventions. Even the pursuit of research itself is rendered unusually difficult through the exigencies of war—shortages in equipment and in trained personnel being perhaps the most apparent: it is expediency to which tribute must be paid.

These matters, it must be repeated, are close to all of us and can never be far removed from those whose pursuit of learning makes them acutely aware of the problems that confront us in the present and in the immediate future. We look forward with eagerness and hope to the day of free intercourse among nations, to the restoration of complete intellectual accord with scientists throughout the earth, and to the collaboration once again of our biochemical colleagues abroad in the preparation of forthcoming volumes of the *Annual Review*. May that day be soon.

It would be repetitious to mention again, as we have done in pre-

vious years, the special difficulties that have confronted the writers of these *Reviews*. We can but express on behalf of the readers of these volumes the very sincere thanks that we feel to all who have done their part in helping to ensure an uninterrupted continuance of the series.

To Professor Hubert S. Loring, to our editorial and office assistants, and to Stanford University Press we wish to acknowledge most gratefully the painstaking help that has been given.

H. J. A.
D. R. H.
J. M. L.
C. L. A. S.
J. H. C. S.
H. A. S.

ERRATA

Volume XI, page 124, line 25: *for* LXVII ⇄ LXIX, *read* LXVII ⇄ LXX

Volume XI, page 245, line 12 from bottom: *for* injection, *read* ingestion.

Volume XI, page 296, line 20: *for* inactivated, *read* inactive.

Volume XI, page 695, middle column, lines 20, 21, and 22: *for* Mirsky, A. E., 39, 173, 212, 225, 229, 230, 419, 473, 525, 544, 662, *read*
Mirick, G. S., 662
Mirsky, A. E., 39, 173, 473, 544
Mirsky, I. A., 212, 225, 229, 230, 419, 525

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BIOLOGICAL OXIDATIONS AND REDUCTIONS

By Fritz Lipmann

Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Massachusetts

The Conversion of Oxidation Energy into Phosphate Bond Energy

Correlation between metabolism and phosphate turnover.—Through a subtle analysis of the metabolism of an autotrophic sulfur-oxidizing organism, *Thiobacillus thiooxidans,* Vogler, Umbreit and their collaborators (1, 2, 3, 4) have disclosed a new and striking example of the general occurrence in nature of a linking between oxidation and phosphate turnover. The processes of energy release through oxidation, in this case of sulfur, and of energy utilization could be separated. The oxidative phase was found to be accompanied by phosphate fixation, and the reductive phase of carbon dioxide fixation by a release of phosphate. O'Kane & Umbreit (5) also studied in some detail the more familiar coupling of glucose fermentation with phosphate fixation, using resting cells of *Streptococcus fecalis.* It may be emphasized that, parallel with the fermentative phosphate shift, an accumulation of potassium has been observed in several microorganisms, e.g., in yeast (6) and in *Escherichia coli.*

With radioactive phosphorus as indicator, Taurog, Chaikoff & Perlman (8) demonstrated a dependence of the synthesis of phospholipids on oxidative metabolism in experiments with liver and kidney slices. Likewise, with brain slices (9), glucose increased the turnover four to five times. The permeation of phosphate into brain cells was followed and found largely dependent on carbohydrate metabolism. Analogous results with yeast are reported by Mullins (10).

Efficiency of energy conversion.—The equivalent per mole of carbohydrate oxidized of energy-rich phosphate bonds, designated with ~ph (11), had been determined by Belitzer & Tsibakova (12) and by Ochoa (13) to about 24 ~ph, or 2 ~ph per atom of oxygen consumed. In such determinations fluoride is used to stabilize, more or less effectively, the generated phosphate esters; due to the unavoidable loss through dephosphorylation, these figures are to be considered minimum values. In a new series of experiments Ochoa (14) has determined now, rather reliably, the extent of the loss through dephos-

phorylation to be 30 to 40 per cent; he arrives at an average of 3 ~ph per atom of respired oxygen, or 36 ~ph per mole of carbohydrate. With 11 kcal. per bond (11) this makes an efficiency of 60 per cent for the conversion of available oxidation energy into utilizable phosphate bond energy. The question then arises how such a high yield of phosphate bonds can be explained in terms of a mechanism. Following the metabolic pathway of one half of the glucose passing from pyruvate through the citric acid cycle, five energy-rich phosphate bonds may be derived as follows: Two ~ph on the fermentation pathway from glucose to pyruvate; two ~ph as acylphosphates, with the oxidation of the two α-keto acids, pyruvic and ketoglutaric acids; and one ~ph, with Kalckar's enolphosphate (15), found on oxidation of fumaric or malic acid. Therefore, when one mole of triose passes through the six primary dehydrogenations represented in the citric acid cycle, five ~ph, or about one for every dehydrogenation, may be accounted for on straining present knowledge. A coordination, therewith, of one phosphate bond with every primary step is suggested which would explain one out of three ~ph for one reduced oxygen. The two remaining phosphate bonds conceivably can only be generated catalytically during the passage of the hydrogens from the metabolite at the bottom of the oxidation-reduction scale to oxygen at the top. The passage of the hydrogens occurs through the span of 1.2 volts, corresponding with 57 kcal., between the substrate and the oxygen. The 11 kcal. of a ~ph represent only 0.24 volts, and there is ample space, therefore, for the three 0.24 volt units in the total span of 1.2 volts. They have, however, to be taken off at different potential levels. Consequently, in addition to the first ~ph generated directly on the metabolite, two transformation catalyzers would be required for the second and the third ~ph at two potential levels intermediate between the hydrogen and oxygen potentials.

As an example of a mechanism of catalytic transformation, the parallel operation of a water system and a phosphate system may be considered, e.g., an aldehyde hydrate/acid system coupled with the corresponding aldehyde phosphate/acyl phosphate system. The loading of this transformer would be accomplished by hydrogenation of the carboxyl group of the water system through a pair of hydrogens arriving from the lowest oxidation-reduction level. The aldehyde hydrate thus formed exchanges through equilibration its water for a phosphate. The resulting aldehyde phosphate may then be dehydrogenated to yield acyl phosphate. Therewith a 0.24 volt equivalent is

transformed into a phosphate bond and after its transfer to adenylic acid, the original carboxylic group is regenerated and may enter a new transformation cycle:

$$R \cdot COOH + H_2X \rightarrow RCH(OH)_2 + X$$
$$R \cdot CH(OH)_2 + HO\text{-}ph \rightleftharpoons R \cdot CH(OH)(O\text{-ph}) + H_2O$$
$$R \cdot CH(OH)(O\text{-ph}) + O \rightarrow R \cdot COO \sim ph + H_2O$$
$$R \cdot COO \sim ph + Ad \rightarrow R \cdot COOH + Ad \sim ph$$

It is known that aldehydes, adding either phosphate or water, are dehydrogenated reversibly. However, the potential level of the usual aldehyde/acid pair is rather low for the contemplated function of a transformer. It seems more probable that systems of higher oxidation-reduction potential will perform this function. Most likely they will be found among the dicarboxylic acids of the Szent-Györgyi cycle [cf. Colowick, Welch & Cori (16)].

Mechanisms of oxidative fixation of phosphate.—The observation that a fixation of phosphate may occur as the result of dehydrogenation of carbonyl compounds necessitated the assumption that an addition product was formed primarily between the carbonyl group and phosphate. Such an addition of phosphate on the carbonyl double bond, however, was an unknown reaction. One of the encountered cases of oxidative phosphorylation had been the oxidative decarboxylation of pyruvic acid (17):

$$CH_3CO \cdot COOH + HO \cdot ph \rightleftharpoons CH_3C(OH)(O \cdot ph)COOH - 2H \rightarrow CH_3COO \sim ph + CO_2$$

Baer (18) has found now a very similar oxidative decarboxylation of α-keto acids with lead tetraacetate. This reaction takes place only with an hydroxyl compound present which, with the carbonyl, can form a half acetal-like addition product in the same manner as postulated for phosphate. This oxidation is interpreted as a glycol splitting (Criegee reaction) in which the half acetal and the neighboring carboxyl group react as a glycol:

$$R \cdot C{:}O \cdot COOH + HO \cdot X \rightleftharpoons R \cdot C(OH)(O \cdot X) \cdot COOH - 2H \rightarrow R \cdot COO \cdot X + CO_2$$

In this reaction oxidative decarboxylation is coupled with esterification in an analogous manner as enzymatic oxidation is coupled with phosphorylation. In a very smooth reaction acetylcholine was ob-

tained by coupled oxidation of pyruvic acid and choline (19), and it is announced that the chemical analogue of enzymatic acetylphosphate condensation has been obtained with this modification of the Criegee reaction.

Continuing the analysis of the enzymatic coupling of pyruvate oxidation and phosphorylation, Lipmann (20) has isolated monoacetylphosphate, the primary oxidation product in the enzyme reaction of *Lactobacillus delbrückii,* as a disilver salt in pure state. Improvements of the method for determination of acetylphosphate and a procedure for direct synthesis of monoacetylphosphate from silver phosphate and acetylchloride were worked out (21).

Koepsell & Johnson (22) have discovered another case where acetylphosphate is intermediate in pyruvate breakdown. They found in extracts prepared from *Clostridium butylicum* an enzyme system which decomposes pyruvic acid to acetic acid, hydrogen, and carbon dioxide. Formic acid, frequently considered an intermediate in this "hydroclastic" reaction, was not attacked. The reaction appeared similar to pyruvate dehydrogenation in *Lb. delbrückii* in that the accessibility of pyruvate for the catalytic splitting depended on the presence of inorganic phosphate. With ordinary methods for phosphate determination no phosphorylation was detected. However, with the method used by Lipmann (17) for determination of acetylphosphate, the accumulation of a labile phosphate compound was observed (23). Furthermore, a coupling of pyruvate decomposition with glucose phosphorylation could be demonstrated, as well as a transphosphorylation from acetylphosphate to glucose (24).

These findings make it almost certain that there is occurring here a reaction to be described most adequately as a "phosphoroclastic" splitting of pyruvic acid:

$$CH_3CO \cdot COOH \ldots . HO \cdot ph \rightarrow CH_3CO \cdot O \sim ph + H_2 + CO_2$$

As pointed out by Koepsell & Johnson (22), the energy residing in the bond between acetyl and phosphate, about 11 kcal. (11), must be derived from the splitting. The reaction then becomes a controlled explosion of the pyruvate molecule with much or all of the mobilized energy passing into the carboxyl-phosphate bond. Through storage of the energy therein, the reaction may be essentially reversible, which seems indicated by the inhibitory effect of hydrogen on butyl fermentation in general (25) and on this partial reaction in particular (24).

Meyerhof & Junowicz-Kocholaty (26) found that there is a two-way interaction between ph~pyruvate and ph-glycerate:

$$\text{ph-glycerate} \rightleftharpoons \text{ph}\sim\text{pyruvate} \qquad (a)$$

$$\text{ph-glycerate} + \text{ph}\sim\text{pyruvate} \rightarrow \text{ph-glyceryl}\sim\text{ph} + \text{pyruvate} \qquad (b)$$

Reaction (*b*) had previously been overlooked because of the instability of the carboxylphosphate (27). This reaction should contribute to the synthesis of ph-glyceryl~ph which leads eventually to the reductive synthesis of carbohydrate (28):

$$\text{ph-glycerate} + \sim\text{ph} + \text{H} \cdot \text{DPN}^{1} \rightleftharpoons \text{glycogen} + \text{ph} + \text{DPN}^{+}.$$

Hydrogenase

During the year, reports appeared on the presence of hydrogenase in green algae (29) and in nitrogen-fixing organisms (30). The demonstration of this enzyme in strictly aerobic organisms seems to indicate a generalized importance of hydrogenase catalysis. Known mainly through the work of Stephenson and her collaborators (31), the enzyme had been observed predominantly in anaerobic organisms.

Nitrogen fixation.—Wilson *et al.* (30) found inhibition of nitrogen fixation by hydrogen and demonstrated a pronounced parallelism between the nitrogen reduction and hydrogenase activity in *Azotobacter* and in nodule bacteria. They obtained solutions of hydrogenase from *Azotobacter* by thoroughly grinding with powdered glass. The solutions also catalyzed the oxyhydrogen reaction and were supposed, therefore, to contain in addition to hydrogenase an oxygen-activating enzyme. The rate of the oxyhydrogen reaction decreased strongly with higher oxygen tensions due to an oxidative inhibition of hydrogenase. This inhibitory effect of high $p\text{O}_2$ seems surprisingly analogous to the oxygen inhibition, described long ago by Meyerhof & Burk (32), of nitrogen fixation and total respiration of *Azotobacter*. Their $p\text{O}_2$ effect may likewise be due to hydrogenase inhibition.

Photosynthesis.—Gaffron (29) found that green algae of the species *Scenedesmus* after several hours of anaerobiosis became able to substitute for normal photosynthesis a photoreduction of carbon dioxide by molecular hydrogen:

$$\text{CO}_2 + \text{H}_2 + \text{light} \rightarrow \text{carbohydrate}$$

[1] DPN = diphosphopyridine nucleotide.

The presence of hydrogenase in these algae was proven through the demonstration of a typical hydrogen fermentation of glucose or endogenous substrates. Furthermore, a photocatalytic hydrogen evolution was demonstrated (33) and was interpreted as photoclastic splitting of water, coupled with the dehydrogenation of a metabolite:

$$2H \cdot OH + H_2X + \text{light} \rightarrow H_2 + 2H_2O + X$$

A third significant phenomenon was found in the hydrogenase-containing algae, i.e., the utilizability in the dark of the energy of the oxyhydrogen reaction for carbohydrate synthesis from carbon dioxide. With these algae in which the hydrogen-activating catalyst acts as a hydrogen detector, Gaffron now approaches the final demonstration that the light reaction in photosynthesis is a photoclastic decomposition of water [van Niel (34)] and that the carbohydrate synthesis from carbon dioxide is essentially a dark reaction.

Chemistry of hydrogenase.—The catalysis of the exchange between deuterium and water (cf. 35) was used by Hoberman & Rittenberg (36) in their study of hydrogenase in ordinary suspensions of *Proteus vulgaris* and *B. lactis aerogenes*. Inactivation through oxygenation, a common attribute of the enzyme wherever it is found, was studied in detail. Complete and reversible inactivation occurred when washed suspensions were shaken for twenty-four hours in oxygen. The inactive enzyme was reactivated only very slowly by hydrogen alone, whereas reactivation was greatly accelerated through addition of hydrogen-donating metabolites.

Through the results of inhibitor studies the assumption seems now well-founded that hydrogenase is an iron compound. The most convincing proof that it contains iron is the observation of a light-reversible inactivation of the reduced enzyme with carbon monoxide (36). This view is further confirmed through a blocking by cyanide of the reactivation of the oxidized enzyme (29, 30). An independence of the catalysis of hydrogen activation and of hydrogen transfer is indicated through the action of urethane which inhibits the hydrogen transfer to methylene blue but not the catalysis of the exchange between deuterium and water (36). In summary: hydrogenase most probably is an iron compound, active in the ferrous but inactive in the ferric form. This makes it highly probable that the enzyme does not undergo a valency change in the process of hydrogen inactivation.

Of interest is the reversible oxidative inactivation of an iron enzyme. The simplicity of the reaction catalyzed by this enzyme made it

possible to recognize with clarity the behavior of a catalyst, the like of which had been suspected for some time, as a transmitter in the more complicated Pasteur reaction (37). As pointed out by Gaffron (29) the cytochrome-like "Pasteur enzyme" (38) may behave comparably to hydrogenase.

Iron-Porphyrin Proteins

Peroxidases.—Theorell, pursuing his admirable work in this field, reports in a number of papers the isolation and analysis of horse-radish peroxidases I and II. Peroxidase II, i.e., the common horse-radish enzyme, was obtained in crystalline form (39) and was studied in great detail. An important step in purification was the electrophoretic separation of the two peroxidases at pH 7.6 where II migrates anodically and I in the opposite direction. The crystallization took place in ammonium sulfate solution as the saturation was raised slowly from 48 to 62 per cent. The crystalline material behaved as an homogeneous compound when studied by cataphoresis, ultracentrifugation, and solubility determinations. For the molecular weight the following figures were obtained: 44,000 when calculated for one hemin per mole (1.48 per cent as chlorhemin), and, in excellent agreement, 44,100 by ultracentrifuge analysis (40), proving peroxidase to be a monohemin protein. The catalytic activity of Theorell's crystalline preparations showed slight fluctuation, a "P.Z." between 900 and 1200. Higher values reported in the literature were discussed and considered due to error.

Horse-radish peroxidase gives three distinctly different compounds with hydrogen peroxide (41) in the following order: a greenish compound with one strong absorption band at 655 to 660 mμ; and two red compounds with two absorption bands at 560 and 530 and at 583 and 545 mμ, respectively. The green compound which forms with low concentrations, and this one alone, is catalytically active. With the finding that on increasing the hydrogen peroxide concentration, a formation of new, apparently inactive compounds occurs, the common experience that peroxidase is strongly inhibited by moderate hydrogen peroxide concentrations finds now an explanation.

With a newly constructed microapparatus for determination of magnetic susceptibilities (42), a detailed study of the magnetic properties of peroxidase II was undertaken (40). The following classification is proposed for iron porphyrin compounds according to magnetic and spectroscopic properties:

1a. *Ferric compounds with essentially ionic bonds.*—These, characterized by strong paramagnetism, are usually brown to green in color with clear absorption in the red. In this group belong peroxidase, the green H_2O_2-peroxidase, horse liver catalase, and methemoglobin.

1b. *Ferric compounds with predominately covalent bonds.*—Weakly paramagnetic, these are red compounds with one to two flatter bands in the green. They are: cytochrome-*c*, the CN-compounds whenever they are formed, e.g., of peroxidase, catalase, methemoglobin, etc.

2a. *Ferrous compounds with essentially ionic bonds.*—These possess strong paramagnetism and are purple red with an absorption band at about 560 mμ and faint additional bands. The group includes hemoglobin and ferroperoxidase.

2b. *Ferrous compounds with essentially covalent bonds.*—These are diamagnetic and of brilliant red color with two well-defined bands in the green. In this group are found oxyhemoglobin, ferrocytochrome-*c*, and all known CO-compounds (e.g., CO-hemoglobin, CO-ferroperoxidase, etc.).

The second peroxidase, paraperoxidase or peroxidase I (39), absorbs at 575, 540, 420, and 370 mμ. Its prosthetic group is also protohemin. The enzyme was found to be rather unstable and was not always present in horse-radish extracts.

Gjessing & Sumner (43) replaced the natural protohemin, i.e., Fe-protoporphyrin, in horse-radish peroxidase with various other metal-porphyrins. They utilized Theorell's method (44) to split the enzyme reversibly through treatment with acid acetone. Mn-protoporphyrin gave with the protein residue a manganese peroxidase with an activity 28 per cent that of the corresponding iron compound. With this ingenious procedure a "synthetic" enzyme of double the activity of the natural enzyme was obtained by exchanging the Fe-protoporphyrin against Fe-mesoporphyrin where the vinyl double bonds are hydrogenated.

In continuation of their work on cytochrome-*c* peroxidase, Abrams, Altschul & Hogness (45) have now obtained nearly pure preparations. The prosthetic group again is protohemin. The isolated ferri compound is brown and absorbs at 620, 500, and 410 mμ. The main bands of ferrous peroxidase are at 560 and 438 mμ. Saturation with hydrogen peroxide turns the brown compound to red, with bands at 560, 530, and 420 mμ. It was shown spectrophotometrically that only

one mole of hydrogen peroxide is bound per mole of hemin. In the purest preparation the equivalent weight for hemin was 60,000. The similarity between the spectra of cytochrome peroxidase-H_2O_2 and oxyhemoglobin is pointed out, and no decision is made as to whether the H_2O_2-compound is in the ferri or in the ferrous state. The absorption of this compound corresponds surprisingly with that of one of Theorell's red H_2O_2-peroxidases. There, however, the red follows a green compound as a second reaction product of H_2O_2 and enzyme.

The demonstration of a peroxidase specific for cytochrome-*c* suggests a wider distribution of peroxidases and, at the same time, a more important role of hydrogen peroxide in biological oxidations. Huszak (46), in Theorell's laboratory, found that the medulla of the suprarenal contains an unusual pattern of oxidation enzymes. Indophenol and paraphenylene diamine were oxidized only with hydrogen peroxide, not with oxygen. Apparently a peroxidase alone is present here. Extracts rich in peroxidase activity were obtained from this tissue. In contrast, the suprarenal cortex contains the normal set of cytochromes together with cytochrome oxidase.

Catalase.—In a modification of earlier methods, Dounce (47) used alcohol and acetone instead of dioxane for crystallization of catalase from lamb and beef liver. According to Greenstein & Andervont (48) the activity of liver catalase is decreased, almost without exception, in tumorous animals. In other organs catalase activity (49) was found to be normal.

Cytochromes.—Bach, Dixon & Keilin (50) find in yeast a new cytochrome, cytochrome-b_2, with absorption bands at 557 and 530 mμ for the reduced compound. Like "b" this cytochrome is slightly autoxidizable and gives protohemin on denaturation. In a subsequent note Bach, Dixon & Zerfas (51) report that this cytochrome follows persistently with preparations of lactic dehydrogenase from various yeasts. Their purest dehydrogenase preparation had a turnover number of 3100 per minute. This preparation did not contain flavin. It reacted with methylene blue but not with cytochrome-*c*. Traces of lactic acid reduced the cytochrome-b_2. They imply that cytochrome-b_2 is either the carrier for the enzyme or is, itself, lactic dehydrogenase. If the latter were true, it would be the first cytochrome with dehydrogenase function. Haas, Hornecker & Hogness (52) point to their earlier description of a hemin, present in impure enzyme preparations, for which absorptions at 557 and 420 mμ (reduced) were indicated and which they consider to be identical with cytochrome-b_2. They re-

port that this compound is reduced by hexose-6-phosphate dehydrogenase plus hexose-6-phosphate.

Melnick (53), continuing work with the indirect spectrophotometric method of Warburg, determined the absorption spectrum of CO-cytochrome oxidase in heart muscle. The main absorption bands were found at 589, 510, and 450 mμ. The distribution of cytochrome oxidase in *Arbacia* eggs was determined (54). In bull spermatozoa (55) the oxidase was found exclusively in the tail and there in rather high concentration. The cytochrome oxidase content of chick embryos (56) was charted at different stages of development. Fischer & Wecker (57) synthesized *Spirographis* porphyrin, suspected to be the porphyrin in the prosthetic hemin of cytochrome oxidase (58).

In a systematic study of the spectra of ferro hemochromogens, Drabkin (59) finds an analogy between the cytochrome-*c* spectrum and that of ferro mesoporphyrins (reduced vinyl groups). This observation furnishes independent confirmation of the view of Theorell that in cytochrome-*c* the vinyl double bonds are desaturated through addition of —SH. A spectrophotometric micromethod for the determination of cytochrome-*c* was described by Potter & DuBois (60). The content of cytochrome-*c* in tumors was found to be low (61).

Hemoglobin.—A series of papers have appeared dealing with the interaction of the four hemes in the hemoglobin molecule. Taylor & Hastings (62) and Taylor (63) made a study of the relation between the number of hemes per mole and the equivalent factor "*n*" as measured by the slope of the oxidimetric titration curve. For myoglobin with one heme (E'_0 pH 7, 0.041 volt), the factor is one. For urethane-hemoglobin, considered a two-heme molecule, the factor two was found, indicating a somewhat unexpected fusion of the two hemes into a two-electron system (E'_0 pH 7, 0.108 volt).

For hemoglobin with four hemes (E'_0 pH 7, 0.144), *n* was found to shift from one to two around the midpoint of the titration curve. In hemoglobin, in other words, with a predominance of ferroheme in the average molecule, *n* is almost exactly one but changes to two when ferriheme becomes predominant. Reference may be made here to the interesting oxidimetric curve for the presumably quadrimolecular pentacyano-aquo complexes of iron [Michaelis & Smythe (64)].

Darling & Roughton (65) report a decrease of hemoglobin-O_2 dissociation by admixture of methemoglobin, either direct or by partial oxidation. They attribute this to a tightening of the linkage between hemoglobin and oxygen in partially oxidized hemoglobin mole-

cules and discuss the physiological consequences of methemoglobin formation *in vivo* resulting therefrom. Cox & Wendel (66) studied the spontaneous reduction of methemoglobin in blood. Granick (67) observed that the dissociation of oxygen from a suspension of oxyhemoglobin crystals is surprisingly slow. The formation of sulfhemoglobin *in vivo* is described by Barkan *et al.* (68).

With radioactive iron as an indicator Ruben *et al.* (69) found that the iron in hematin and methemoglobin is nondissociating. They point out that the intramolecular ionization of the iron, indicated by paramagnetism, is not to be mistaken for dissociability. The dielectric properties of monoheme myoglobin and four-heme hemoglobin were compared by Marcy & Wyman (70). Lemberg *et al.* (71) reported a compound, choleglobin, formed by coupled oxidation of hemoglobin and ascorbic acid, which they regard as a complex of an iron-bile pigment with native globin.

Non-Hemin Iron and Other Heavy Metal Proteins

Warburg & Christian (72) describe the inactivation of yeast zymohexase by 0.02 molar cysteine, pyrophosphate, and cyanide. Activity could be regenerated entirely through addition of 10^{-5} to 10^{-4} molar zinc, cobalt, and iron. They furthermore observe that oxygen inhibits reversibly the reactivation by cobalt and iron, but not by zinc. This effect of oxygen is considered due to oxidation of active divalent to inactive trivalent iron or cobalt. They conclude that a divalent heavy metal functions as the dissociable prosthetic group in zymohexase. Zymohexase preparations from muscle were not inactivated by complex-forming anions. The explanation is proposed that in the muscle enzyme the metal is more tightly bound than in the yeast enzyme.

Ferretin, a crystalline protein from spleen with 23 per cent iron, was studied by Granick & Michaelis (73). One atom of iron, it is pointed out, corresponds here to approximately one peptide bond. The iron seems loosely bound: after reduction with sodium hydrosulfite, it could be removed from the ferretin crystals by γ-γ-dipyridyl without change of shape of the crystals. The magnetic properties were found similar to those of methemoglobin and catalase. A relation of this compound to the process of blood formation is surmised through the finding that after bleeding, the amount of ferretin in the spleen was decreased to only 10 per cent of the normal content.

An amorphous protein with 0.19 per cent undialyzable copper was isolated (74) from milk. No function could be assigned to this new copper protein. Two groups of workers (75, 76) have attempted to decide the question of whether hydrogen peroxide is formed in enzymatic ascorbic acid oxidation. Both reached the conclusion that it is not formed. The interesting fact, however, is reported (76) that the enzyme, which in activity is rapidly destroyed, is most effectively protected by catalase and less by peroxidase and methemoglobin.

Interesting, although inconclusive, results were reported by Davidson (77) in further work on uricase. His purest preparations contain both iron and zinc in large amounts. Other metals were found only in traces. A mapping of the pO_2 function showed that at 100 per cent oxygen tension the rate was far from maximum. Potassium thiocyanate, considered a more or less specific inhibitor for zinc, reacted in a peculiar way: an inhibition appeared first after five to twenty minutes incubation with the substrate. Allantoin, as the genuine product of oxidation with uricase, was confirmed through isolation, in good yield, from uric acid oxidation with highly purified enzyme preparations.

Tyrosinase.—A connection between tyrosinase and/or peroxidase activity and thyroxine synthesis *in vivo* may be suggested by recent work on the antithyroid effect of thiourea and derivatives and of large doses of sulfonamides. Inhibition of mushroom tyrosinase by phenylthiocarbamide was found by Bernheim *et al.* (78), and inhibition of peroxidase by sulfonamides had been described (79). Thiourea and derivatives were found to inhibit peroxidase and a good, but not perfect, parallelism between this inhibition and antithyroid activity was observed [Astwood (80)]. Westerfeld *et al.* (81) discuss the possibility that the quinol-ether linkage in thyroxine may be formed through a peroxidatic condensation. He found a quinol ether among the oxidation products of paracresol by peroxidase.

A depigmentation of the human skin, brought about by the monoethyl ether of hydroquinone, induced Peck & Sobotka (82) to study the action of this compound on tyrosinase. They found that with tyrosine as substrate the oxygen consumption was not diminished but the course of oxidation was changed in such a manner that the primarily formed reddish compounds of orthoquinone type failed to undergo the condensation which normally leads to melanin formation.

Hogeboom & Adams (83), suspecting large amounts of tyrosinase in melanotic tumors, studied melanoma tissue and describe it as by

far the most abundant in tyrosinase of all tested mammalian tissues. An enzyme similar to laccase had been found in acid extract from kidney (84). Zondeck *et al.* (85) consider the enzyme in potato juice, responsible for inactivation of estrone, to be similar to but not identical with polyphenol oxidase. Blaschko (86) studied the dopa decarboxylase of animal tissues, especially of kidney. He discusses the possible role of this decarboxylation in tyrosine breakdown, for which he suggests a pathway over hydroxytyrosine followed by decarboxylation to hydroxytyramine.

Nicotinamide, Thiamin, and Flavin Enzymes

Pyridine nucleotides.—The formula of cozymase may now be considered as definitely established. Although there was no reasonable doubt as to the general trueness of the picture, the unambiguous identification of the two moles of carbohydrate in the molecule was still missing. Schlenk (87) and Euler, Karrer & Usteri (88) have now independently given proof that each of these carbohydrates is a *d*-ribose and that each has phosphoric acid attached in 5-position.

A number of informative observations were made on the breakdown and synthesis of phosphopyridine nucleotides in tissues and microorganisms. Handler & Klein (89, 90) followed the destruction of diphosphopyridine nucleotide (DPN) by tissue enzymes with the V-factor test and, at the same time, determined nicotinamide colorimetrically. With a brain suspension of 0.4 gm., 0.5 mg. DPN were decomposed in ten minutes, yielding 97 per cent of the possible amount of nicotinamide but no free phosphate. It is suggested that a cleavage of DPN takes place at the nucleosidic linkage between nicotinamide and ribose. Approaching the biological mechanism of DPN synthesis, Schlenk & Gingrich (91) found that a fragment of DPN, the nucleoside nicotinamide-*d*-ribose, could replace very effectively DPN in the organisms needing the V-factor; they suggest that the linkage in DPN of nicotinamide to ribose is the reaction missing in these organisms. It occurred to us that this condensation may be accomplished in the cell by way of phospho-1-ribose which would act as the biological analogue to a Br-1-aldose. Acetobromoglucose is frequently used in the laboratory for similar condensations.

The earlier reported stimulation of succinoxidase by calcium is now explained by Swingle, Axelrod & Elvehjem (92) as being due to stimulation of the nucleotidase which splits DPN. It is known that

oxaloacetate poisons succinoxidase in minimal concentrations. The formation of oxaloacetate from fumarate-malate requires DPN as a catalyst in the system. Thus, the stimulation by calcium seems to be a protection from poisoning through removal of the catalyst needed for the production of the poisonous oxaloacetate. It remains to be seen if the effects of aluminum and chromium ion on succinoxidase (93) may not find a similar explanation or may be due to participation in the enzyme reaction proper.

The fate of nicotinic acid in the animal body was studied by Huff & Perlzweig (94). They report that at least 30 per cent of the ingested nicotinic acid is excreted as trigonelline by man, dog, and rat.

A new pyridine enzyme seems suggested through the work of Klein & Handler (95). They found in washed liver tissue an activation by DPN of the oxidation of betaine aldehyde to betaine. No effect on choline oxidation was observed. Jandorf & Krahl (96) reported in *Arbacia* eggs a remarkably high concentration of DPN, i.e., 0.25 to 0.5 mg. per gm. wet weight, which is nearly as high as in muscle. Torpova (97) studied the equilibria between lactic, pyruvic, and other α-keto acids on lactic dehydrogenase from heart muscle.

Thiamin enzymes.—Green *et al.* (98) published an extensive report of their isolation of thiamin enzymes from animal tissues. A suspensoid solution containing these enzymes was obtained from the residue of washed pig heart by extraction with glycerophosphate of pH 6. Three separate enzymes were distinguished which catalyzed, respectively: (*a*) the straight decarboxylation of α-ketoglutarate to succinosemialdehyde; (*b*) a decarboxylation of pyruvate which is remarkably accelerated by acetaldehyde and is rigidly coupled with acetoin condensation; and (*c*) a straight condensation of two aldehydes to acetoin. The latter reaction is of special interest as it represents the first observation of a nondecarboxylative thiamin catalysis. The functioning of thiamin in various, more or less complex condensation reactions had been outlined by Barron *et al.* (99) . The enzymatic reactions of Green are exclusively anaerobic reaction. Oxygen is without influence and phosphate is not involved. It may be of significance that arsenite is without action on these decarboxylations in contrast to its inhibition of α-keto acid oxidation in animal tissues. As an outgrowth of this work, it was considered by Westerfeld *et al.* (100) that a condensation to acetoin might be demonstrated by furnishing pyruvate to the aldehyde formed through alcohol oxidation. An acceleration of alcohol disappearance was actually found with the simul-

taneous intake of pyruvate. This was shown, however, to be due to a dismutation between alcohol and pyruvate which yielded aldehyde and lactate (101). However, acetoin and diacetyl may be formed and metabolized in tissues.

Krampitz & Werkman (102) have isolated β-decarboxylase, the enzyme catalyzing decomposition of oxaloacetate. In a later paper (103) it is announced that the same enzyme catalyzes β-carboxylation, i.e., the synthesis of oxaloacetate from pyruvate and carbon dioxide (Wood and Werkman reaction). The enzyme was found in preparations of *Micrococcus lysodeicticus,* where it is accompanied by an enzyme which oxidizes pyruvate. In order to characterize the enzyme and to test for the alleged (104) role of cocarboxylase, acetone preparations were washed with alkaline phosphate to remove cocarboxylase. The decarboxylation of oxaloacetate then required the addition of magnesium but not of cocarboxylase, whereas oxidative decarboxylation of pyruvate required both. Thereby it is shown that the decarboxylation and carboxylation in β-position are not thiamin-catalyzed reactions, and the claim of Krebs (104) that the reaction which is missing in thiamin deficiency is mainly the synthesis of oxaloacetate becomes invalid. Evans, Slotin & Vennesland (105) obtained cell-free liver preparations which catalyzed carboxylation.

Quastel & Webley (106) studied further the thiamin effect on acetate oxidation observed in thiamin-deficient propionic acid bacteria. Evidence is presented that the thiamin effect is increased through previous incubation with metabolites which further its phosphorylation. Wirth & Nord (107), working with *Fusarium bolley,* describe an accumulation of pyruvate which resulted from the action of nitrate on glucose or pentose fermentation. Thiamin, when added in excess, prevented the accumulation of the intermediate. The interpretation is given that the nitrite formed on reduction of nitrate may partly inactivate carboxylase.

Nachmansohn *et al.* (108) furnish indirect support for the theory that thiamin enzymes are involved in the synthesis of acetylcholine. In the giant nerve fiber of the squid, they found an accumulation of cocarboxylase in the surface layers where a rapid formation of acetylcholine may be expected to take place. Krahl *et al.* (109) found 2 to 3 μg. per gm. wet weight of cocarboxylase in *Arbacia* eggs and found the same for fertilized and unfertilized eggs.

Flavin enzymes.—Haas, Harrer & Hogness (110) further purified the yeast flavoprotein, designated cytochrome reductase, which is

a link between triphosphopyridine nucleotide and cytochrome-*c*. They studied in detail the comparatively slow reaction with oxygen. Although this flavoprotein was found to be inhibited by dinitrophenol, a strict specificity of dinitrophenol inhibition for flavoproteins, as suggested by Krahl *et al.* (111), was denied for the reason that the pyridine-catalyzed dehydrogenation of hexose-6-phosphate was even more strongly inhibited than the flavin enzyme. Axelrod *et al.* (112) find in flavin-deficient rats a decrease of succinoxidase in the liver. They suggest that a flavoprotein may be a link in this oxidase system.

Holtz & Buechsel (113) and Karrer *et al.* (114) continue their work on the specificity of *d*-amino acid oxidase. The inactivation of *d*-amino acid oxidase through irradiation with x-rays is studied by Dale (115). A remarkable synthesis of flavin adenine dinucleotide from added riboflavin with muscle tissue is described by Trufanov (116). Between 1 and 2 mg. could be formed in five to six minutes, and oxygen is required for the synthesis.

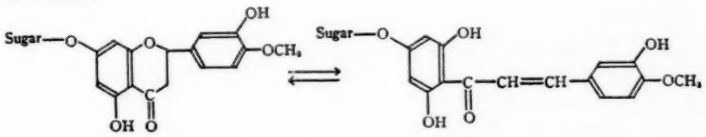
The evidence that diamine oxidase may be a flavin enzyme is summarized by Zeller (117). Laskowski (118) purified diamine oxidase and, contrary to Zeller, found no indication that hydrogen peroxide is formed with this oxidase.

Enzyme Functions Attributed to Other Vitamins

Pantothenic acid.—Evidence is accumulating that pantothenic acid is part of an enzyme involved in oxidative metabolism. Dorfman *et al.* (119) showed with deficient cells of *Proteus morganii* that the addition of pantothenate to resting suspensions increased oxidation of lactate and pyruvate two- and threefold. Both were oxidized essentially to acetic acid and carbon dioxide. It is tentatively assumed that pantothenic acid is more or less specifically concerned with the process of pyruvate oxidation. Pilgrim *et al.* (120) find pyruvate oxidation decreased in the homogenate of livers from rats deficient in pantothenate or biotin. Much of the pantothenate in yeast and maceration juice from yeast is shown by Teague & Williams (121) to be present in a protein-bound form. Experiments to show the metabolic effects of the addition of pantothenate to maceration juice and tissues from deficient chicks were unsuccessful.

Vitamin P.—The mixture of hesperidin and "eriodictin," which represents Szent-Györgyi's vitamin P, was found by Wawra & Webb

(122) to be an equilibrium mixture of hesperidin and hesperidin chalcone:



The yellow chalcone was found to be reduced by dehydrogenase action and reoxidized by atmospheric oxygen. The chalcone may, therefore, be able to function as a hydrogen carrier. It combines easily with proteins and was isolated from lemon peel in the protein fraction—perhaps, in genuine protein linkage.

Tocopherol (vitamin E).—An increase of the *in vitro* respiration of muscle strips from vitamin-E-deficient rodents in the stage of incipient atrophy had been observed some years ago by Victor (123). Houchin & Mattill (124) have found now that the increased respiration is returned to normal levels by addition *in vitro* of α-tocopherol phosphate. They found, furthermore, that succinoxidase preparations from muscles with an average Q_{O_2} of fifty were brought down to an average and normal value of nineteen on addition of the tocopherol phosphate. Nonphosphorylated tocopherol gave inconclusive results. The nonphosphorylated compound is, however, extremely insoluble in water while the phosphate is relatively soluble. It is considered possible that the effect of the phosphorylated compound is due to increased solubility rather than to specificity. In the discussion of these results the increase of respiration in the deficient muscle is tentatively attributed to its inability to hold respiration at a resting level in the absence of the vitamin.

Oxidative Inactivation of Enzymes

Proteolytic enzymes.—An interesting aerobic inhibition in spleen and kidney preparations is reported by Irving, Fruton & Bergmann (125) who found sulfhydryl-activated proteases and peptidases to be partially and reversibly inactivated by oxygen. This inactivation, however, is not due to oxidative destruction of the activating sulfhydryl compound as this was present in excess and did not disappear appreciably during the reaction. Fractionated but still rather complex tissue preparations were used for these experiments. On changing from nitrogen to oxygen there was observed a short induction period

when inhibition developed full strength. Inhibition was most pronounced in citrate buffer, less in phthalate, and absent in acetate buffer. Experiments with highly purified papain under equal conditions showed no inhibition.

The inhibitory effect of oxygen on autolysis in hog liver suspension was studied by Bailey *et al.* (126) and was attributed here to oxidation of the activating sulfhydryl compounds. Inhibition was likewise obtained with potassium iodate or other oxidants. A synthesis of protein in oxygenated suspension was not observed.

The effect of quinones and quinone imines.—Kensler *et al.* (127) started an investigation of the action of phenylenediamines on enzymes because these compounds had been found among the split products of the cancerogenous dimethylaminoazobenzene (butter yellow). They demonstrated an inhibition of fermentation which was especially pronounced with highly methylated diamines. Washed dry yeast, recombined with cozymase, was used as the test system. This inhibition was dependent on the presence of oxygen, and cysteine or ascorbic acid could protect the enzyme. The inhibitory effect was, therefore, considered to be caused by oxidation products of the paraphenylene diamines, presumably of a quinone or semiquinone type. Parallels were found to the earlier observed oxidative inhibition of fermentation by indophenols, quinone, and iodine (128, 129, 130) and to the work of Rapkine (131) on oxidation of the sulfhydryl groups of triphosphate dehydrogenase. In a second paper on this subject (132), an analogous inhibition of carboxylase was described. Data are given which show quite a pronounced stimulation of carboxylase by cysteine, which was also seen by Cajori (133).

Stimulated by a suggested relation between these inhibitions and the mechanism of cancer induction, this type of inhibition has been studied diligently and was found with a great variety of enzymes. Already, Ball (134) had become aware of a destruction of succinic dehydrogenase when an oxidation of *p*-phenylenediamine by the cytochrome oxidase of the enzyme system had taken place. Potter (135) found irreversible inhibition of urease and gives preliminary data on the effect on succinoxidase. He discusses the general mechanism of this inhibition and points out that for a given compound, activity depends on two independent factors: (*a*) the rate at which the oxidation product is formed and (*b*) the inhibitory activity proper of the oxidation product. Cohen *et al.* (136) described the oxidative inhibition of transaminase. Friedenwald & Herrmann (137) found that

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amine oxidase is inactivated by quinones formed through oxidation of polyphenols by polyphenoloxidase. Inactivation of yeast invertase by strong oxidants was described by Sizer (138). The effect is irreversible. He (139) also studied the oxidative inhibition of alkaline kidney phosphatase which was to some extent reversible. Partial recovery was obtained by dialysis or treatment with sodium sulfide. Since ferricyanide was ineffective, this inhibition may be due to groups other than sulfhydryl.

Pasteur reaction.—Runnström & Brandt (140) found that the balance between breakdown of polysaccharide and synthesis [cf. Barker (141)] from a limited amount of glucose was significantly changed by glutathione. The normal relation of oxidation: polymerization: aerobic glycolysis of 4:6:0 is changed to 4:0:6—aerobic glycolysis taking the place of polymerization. This anti-Pasteur effect is interpreted as a switching of glucose-phosphate from glycogen synthesis to fermentative breakdown. Gottschalk (142) proposed a competition of oxidants for the reduced DPN as an explanation of the Pasteur effect [cf. also (29), (30), (36), (37), (72)].

Fatty Acid Oxidation

Unsaturated fatty acids.—The oxidation of compounds of the oleic acid type by lipoxidase from soy beans was investigated independently by Sumner (143) and Süllmann (144, 145). In many respects these investigations complement each other and may be reported jointly. Sumner followed the reaction by oxidimetric determination of formed peroxide, whereas Süllmann measured oxygen consumption. With both methods, an increase in activity with the increasing number of double bonds was observed. It seems from the oxygen uptake that only one of the two double bonds in linoleic acid and two of the three in linolenic acid were oxidized. Indications were found that carbonyl compounds were among the oxidation products (145). The coupled oxidation of carotene occurs only concomitantly with fatty acid oxidation although large amounts of peroxide may accumulate. Slightly less peroxide is formed in the presence of carotene, and oxygen consumption may even be decreased. The effect on carotene is reminiscent of the oxidation of ethanol through catalase; according to Keilin *et al.* (146), this does not take place directly with hydrogen peroxide but only when another enzyme reaction yields hydrogen peroxide.

Succinic acid is the only "fatty" acid whose breakdown mechanism is well established; the three enzymatic steps leading to the corre-

sponding keto acid, i.e., oxaloacetic acid, are: desaturation, hydration, and dehydrogenation. As is well known, butyric, crotonic, and hydroxybutyric acids are easily oxidized to acetoacetic acid (147). Therefore, a mechanism analogous to that for succinic acid has been suggested in spite of evidence to the contrary. Experiments by Lipmann & Perlmann (148) on the crotonic acid metabolism of animal tissues and tissue extracts show, however, quite definitely that the succinic acid pattern is not applicable in this case because no link between crotonic and hydroxybutyric acid could be found. In the course of these experiments an anaerobic disappearance of crotonate, as yet unaccounted for, was observed in kidney but not in liver homogenates.

Acetoacetic acid.—The possibility of an enzymatic splitting of acetoacetate to acetate was carefully investigated by Lehninger (149). His conclusions are that a small, irregular, but definite splitting takes place in kidney and muscle tissue and that in *Esch. coli,* large quantities of acetate may be formed from acetoacetate. In experiments on a smaller scale, Stadie *et al.* (150) found that none or insignificant amounts of acetate were formed in liver slices during keto-body formation.

The synthesis of acetoacetate from acetate with *Cl. acetobutylicum* is dependent on the presence of donors of "active" acetate, like pyruvate and glucose [Davies (151)]. Swendseid *et al.* (152) found that in experiments with heavy carbon the fed acetate appeared predominately in the carboxylic part of acetoacetate. Finally, the finding of acetylphosphate as an intermediate in the splitting of pyruvic acid with *Cl. butylicum* (22) is mentioned in this place because it promises new approaches to the understanding of acetoacetate synthesis and breakdown.

Unclassified Enzymes. Methods

Bernheim & Bernheim (153) continued work on the oxidative demethylation of sarcosine to glycine and formaldehyde. They described some characteristics of the enzyme system which is found in the insoluble liver fraction. Tarr (154) described the oxidation of trimethylamine to trimethylamine oxide in the animal body and found trimethylamine oxide to be a normal product of animal metabolism. These papers reveal some phases of the breakdown of methyl groups. The tracing of their fate in the body has become significant in view of du Vigneaud's transmethylation (155) and his demonstration of

the quite limited availability of methyl groups in the animal body (156).

Rosenthal *et al.* (157) charted the dehydrogenase content of cartilage. Friedenwald & Herrmann (158) found that in the choreoid plexus the oxygen-activating enzymes were exclusively in the epithelium but the hydrogen-activating enzymes were in the stroma and epithelium in equal amounts. This unequal distribution between stroma and epithelium is related to the secretory function of the organ. Goepfert & Nord (159) studied alcohol dehydrogenases in *Fusarium* and isolated glycolaldehyde as the dehydrogenation product of ethylene glycol.

Dropping mercury electrode.—Selzer & Baumberger (160) reinvestigated the applicability of the polarographic method for studies on the respiration of living cells. Without stirring, they found that only the low endogenous respiration was measured. With stirring, in a glucose medium, the rate of respiration of yeast was increased ten times. Stirring with mercury, however, led to rapid poisoning. To avoid contact between mercury and the cells a double vessel was constructed. Warren (161) compared polarographic and manometric measurements on myelocytes, the first animal cells to be studied in this manner. Polarographically, the rate of oxygen consumption was constant down to 0.4 per cent oxygen pressure, whereas, with the manometric method with as high a pressure as 3 per cent oxygen, 50 per cent inhibition was observed. Details of the polarographic experiments were not included. Müller (162) compared polarographic and platinum-electrode measurements of the oxidation-reduction potential of oxyphenazine and discussed the question of tautomerism. Oxidation-reduction potential measurements with compounds of the phenazine series were also reported by Eggers *et al.* (163). A polarographic microdetermination of oxygen in small fluid samples was worked out by Beecher *et al.* (164).

Tissue respiration technique.—A critical analysis of the manometric method by Laser (165) has appeared which is highly recommended to all workers using the tissue slice technique. The limited value of isolated measurements of Q_{O_2} is emphasized, especially when carried out in phosphate solutions with the absorption of carbon dioxide. The absence of carbon dioxide was found to cause abnormalities of more qualitative than quantitative nature. It is pointed out that frequently a quantitatively "normal" respiration may be qualitatively grossly abnormal.

Elliott *et al.* (166, 167) report a very useful comparative analysis of the metabolic behavior of brain as homogenate and in slices. In a perhaps somewhat bold manner, the homogenized brain is considered almost equivalent to slices. Trowell (168) describes a relatively simple procedure for the continuous perfusion of a rat liver with saline.

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PROTEOLYTIC ENZYMES

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Introduction

The world conflict has taken its toll of the papers on proteolytic enzymes as it has on nearly everything. There was a gradual decrease in the number of papers appearing during the period covered in this review, namely 1941 and 1942. Some of the foreign journals were not available for obvious reasons and so must await a subsequent review.

Most of the workers have pursued the same general lines of investigation as have been reported in the past. In this review those papers of general or theoretical interest have been presented first followed by those applying more specifically to the individual proteinase and peptidase.

General and Theoretical

Linderstrøm-Lang & Jacobsen (1) have studied the volume changes (contraction) occurring when peptide bonds are broken. They present a theoretical discussion and assume the change to be due to electrostriction. Calculation revealed that one should obtain a change of 17 to 18 ml. for each bond broken per mol of substrate if the side chains of the peptide are not charged and that this value might reach 25 ml. if the side chains are charged. In testing the theory they found that when clupein was acted upon by either of the crystalline enzymes, trypsin or chymotrypsin, the change in volume was in good agreement with the calculated value, but when lactoglobulin was the substrate the high value of 50 ml. was obtained. Haugaard & Roberts (2), on the other hand, digested alkali-denatured lactoglobulin with pepsin at pH 1.5 and obtained the expected value of 24 ml. per bond broken.

A number of papers have appeared which bear on the nature of proteinase action as suggested by Tiselius & Eriksson-Quensel (3), who found upon examining an egg albumin solution during peptic digestion that no intermediate products having a molecular weight above about one thousand were present.

Petermann and associates have demonstrated that pepsin at pH

4.0 to 4.5 does produce intermediate products from diphtheria antitoxin (4), horse anti-pneumococcus antibody (5), and horse or beef pseudoglobulin, and euglobulin (6). In this last paper (6) she finds from ultracentrifugal analyses that the breakdown was apparently into halves and quarters and then into dialyzable fragments. As the pH was shifted toward pH 2.0 (the conditions of maximum digestion rate used by Tiselius & Eriksson-Quensel), the ratio of the components was shifted toward the lighter molecules; *i.e.*, there were fewer halves and quarters, and more of the small fragments. Papain (7), on the other hand, at its maximum digestion rate, pH 5.0, produced the intermediate halves and quarters.

Haugaard & Roberts (2) report that when lactoglobulin was attacked by pepsin at pH 1.5, no fragment insoluble in 10 per cent trichloracetic acid or nondialyzable except for the original protein could be detected, and the amino nitrogen / total nitrogen ratio of the soluble and dialyzable fragments was constant throughout the hydrolysis. Their results tend to confirm those of Tiselius & Eriksson-Quensel (3).

Lundgren (8) concludes from ultracentrifugal, kinetic, and electrophoretic measurements that active papain catalyzes the denaturation of thyroglobulin prior to any hydrolytic cleavage.

By a spectrophotometric examination of the biuret reaction, Sklovskaya & Gorbachevskaya (9) claim to detect disruption in proteins by proteases long before amino or carboxyl groups are detectable. They believe that what they determine is not the disruption of a peptide bond but something which precedes it.

Butler (10) has determined the velocity constants of the following reactions: (*a*) trypsin on benzoyl-*l*-arginine amide, (*b*) trypsin on sturin, (*c*) trypsin on chymotrypsinogen, (*d*) chymotrypsin on benzoyltyrosyl-glycine amide, and (*e*) chymotrypsin on denatured pepsin. The constants were determined at various temperatures and the heats and entropy of activation calculated. The values of all these reactions come reasonably close to the range regarded as that of normal reactions of small molecules. The determined rates are fairly close to the values calculated by the simple collision hypothesis except for the conversion of chymotrypsinogen into chymotrypsin by trypsin which is considerably more rapid than he calculates it to be. Butler suggests that in highly specific reactions, such as these involving large molecules, it is hardly likely that all collisions having the proper energy will lead to reaction. He therefore suggests that there is a compensating factor

such as the formation of a stable enzyme substrate complex which produces an enhanced rate.[1]

Fruton, Irving & Bergmann (11) have found that a number of quite different enzymes (trypsin, papain, and cathepsin) from different sources, although acting at different rates and pH, all have the same proteolytic quotient, Q. Q is the ratio of the first order velocity constants per milligram of protease nitrogen for two synthetic substrates.

Fruton & Bergmann (12) find that crystalline chymotrypsin exhibits several different and distinct specificities on synthetic substrates. It splits *l*-tyrosine amide or *l*-phenyalanine amide, thus making it a typical amino peptidase. It also splits glycyl-tyrosine amide or glycyl-phenylalanine amide at the amide linkage, thus making it a carbonyl proteinase. This is in addition to their original finding that chymotrypsin is an endopeptidase because it splits benzoyl-*l*-tyrosyl glycine amide at the tyrosyl-glycine linkage.

Bergmann & Fruton (13) have also set up a general equation for the action of all proteases on peptides of known structure. Their general equation is: $K = p_E [E]_{abr}$; where K is the reaction velocity constant; p_E, a proportionality factor characteristic of the enzyme "E"; [E], enzyme concentration in mg. protein nitrogen per ml. of test solution. *a, b,* and *r* are numerical factors determined by the structure of the groupings A, B, and R in the substrate. R is defined as the amino acid residue bearing the side chain which is essential for enzyme action. A represents the substituent attached to the α-imido group of R, and B represents the group attached to the α-carboxyl group of R.

The writers suggest that the validity of their proposed equation may be tested by comparing the action of an enzyme upon two substrates which differ only with respect to (*a*) their R groups, (*b*) their A groups, (*c*) their B groups, and (*d*) by comparing the action of two different homo-specific enzymes on the same substrate. This they have done with the swine carboxypeptidases.

[1] The recent findings of Steinhardt [Steinhardt, J., *J. Biol. Chem.*, **141**, 995–96 (1941), Steinhardt, J., and Fugitt, C. H., *Natl. Bur. Standards*, **29**, 315–27 (1942)], although not enzymatic in one sense, have a direct theoretical bearing on catalytic proteolysis. He finds that anions related to dodecyl sulfonate accelerate the hydrolysis of proteins by dilute hydrochloric acid. With egg albumin and wool the rate of acid hydrolysis was increased a hundredfold by 0.05 *M* dodecyl sulfonate.

Individual Enzymes

Papain.—A new crystalline papain has been isolated by Jansen & Balls (14) starting with the mother liquor from the previously described crystalline papain (15). They first removed an inert protein from the mother liquor and then obtained broad sabre-like "needles." The new crystals gave a strong nitroprusside test and were more soluble in certain salt solutions than the first crystalline papain (15). The ratio of the milk-clotting activity of the new enzyme to its hemoglobin activity was twice that of the first crystalline papain so the authors chose to call the new crystalline product, chymopapain. This name may be misleading, for actually the milk-clotting activity per milligram of protein was the same for the two enzymes. It was the hemoglobin activity wherein they differed.

Two other new papain-like proteases have been described: one by Asenjo & Capella de Fernandez (16) from *Bromella pinguin L.* and the other by Castañeda, Gavarron & Balcazar (17) using *Pileus mexicanus*. The former is supposed to digest living tissue and the latter has strong antihelminthic properties.

Very little has been published with regard to the chemical characteristics of papain. Darby (18), however, finds that contrary to the report of Fruton & Lavin (19) tyrosine and tryptophane can be detected in the ultraviolet absorption spectra of the intact papain molecule.

The subject of papain activation[2] has been studied by Irving, Fruton & Bergmann (20) who report that the dialyzable, naturally occurring activator of papain must act in the presence of hydrocyanic acid and that inactivation takes place when the hydrocyanic acid is pumped off *in vacuo*. Addition of hydrocyanic acid then reactivates the enzyme.

The natural activators may be replaced by sulfhydryl compounds such as hydrogen sulfide, cysteine, or glutathione. The activation of dialyzed papain may be represented by the following equation:

$$\underset{\text{(inactive)}}{\alpha\text{-papain}} + H_2S \rightarrow \underset{\text{(inactive)}}{\beta\text{-papain}} \quad \text{(Dialyzed papain)}$$

$$\beta\text{-papain} + \begin{matrix} H_2S \\ \text{or} \\ HCN \end{matrix} \rightleftharpoons \underset{\text{(active)}}{\text{papain}}$$

[2] An article on the activation of papain by Scott, E. M., and Sandstrom, W. M., in the *Arch. Biochem.*, **1**, 103–9 (1942), came to the reviewer's attention after the final draft of the review was made.

Combining the dialyzed-inactive enzyme with the dialysate in the presence of hydrocyanic acid brings full recovery of activity. In all instances the activity is toward the synthetic substrate, benzoyl-arginine amide. Evidence is also presented to indicate that hydrogen sulfide acts on one or more substances to form the activator. The authors suggest that the oxidation-reduction procedures of other workers affect the activators and not the enzyme.

These authors have written two other papers of interest in this connection. They find (11) that papain contains a cysteine-activatable enzyme component having a specificity similar to that of crystalline pancreatic trypsin and they therefore call it "papain-trypsinase." In a later paper (21) they present experiments which bring out the methods of activating this enzyme and show that the rate with which it hydrolyzes benzoyl-arginine amide depends on the activator used to convert the inactive form to the active enzyme. The C value which is the first order velocity constant per milligram of enzyme protein nitrogen was 0.38, and 0.37 when activation was by glutathione or cysteine, 0.30 when by hydrocyanic acid, and 0.12 when by hydrogen sulfide.

Lineweaver & Hoover (22), using crystalline papain, noted that the rate of digestion of hemoglobin is increased by the presence of urea. The rate in 6 M urea was one hundred times that of water. The rates of digestion of hemoglobin denatured by heat, alkali, and neutral urea were the same. They also concluded from these studies that the native hemoglobin was digested by papain. Among other things they found that papain was stable in 9.0 to 10.0 M urea while chymotrypsin was unstable even in 2 M.

Cathepsin.—The major work on this enzyme has been carried out by Fruton, Irving & Bergmann (20, 23), who find that aqueous extracts of beef spleen contain three proteolytic enzymes which they have called Cathepsin I, II, and III.

Cathepsin I does not require an activator and is probably the proteinase purified and described by Anson (24). It has a C value on carbobenzoxy-*l*-glutamyl-*l*-tyrosine of 0.003 where C is the first order velocity constant divided by the enzyme concentration in milligrams protein-nitrogen per milliliter.

Cathepsin II requires an activator such as cysteine. Its C value on *l*-leucine amide, LA, is 0.0094, on benzoyl-*l*-arginine amide, BAA, 0.008. This gives a ratio of C_{BAA}/C_{LA} or Q of 0.8. Upon fractionation of this enzyme preparation the Q value changed to 2.6 which is

evidence that there is more than one enzyme present acting on one or more of the substrates.

Cathepsin III is the enzyme that hydrolyzes *l*-leucine amide in the presence of ascorbic acid. It is activated by cysteine.

Cathepsin I is less stable to heat than the other two enzymes. Evidence is presented indicating that Cathepsin I and a cysteine-activatable enzyme can both hydrolyze carbobenzoxy-*l*-glutamyl-*l*-phenylalanine.

In a later paper (11), these same authors find a fourth enzyme, Cathepsin IV, in beef spleen. They also find the counterparts of these four cathepsins in extracts of beef kidney and swine kidney. In this paper (11) the authors have revised their classification of proteolytic enzymes and now base it on the nature and position of the chemical groups in the substrate peptide chain. They find that a number of quite different enzymes (trypsin, papain, and cathepsin, from different sources), although acting at different rates and pH, all have the same proteolytic quotient, Q.

Freudenberg (25) finds that a considerable portion of gastric juice protease activity is due to cathepsin. The activity of gastric juice is increased by hydrogen sulfide or hydrocyanic acid and has more activity at 60° C. than expected of a pepsin solution. He also finds (26) that the maximum activity of gastric juice toward formed skim milk is at pH 4.0 to 4.5 and only 40 per cent of this value at pH 2.0.

Kies & Schwimmer (27) have determined the amount of catheptic proteinase in brain tissue by its action on hemoglobin. They find that on a protein basis there is twelve times as much cathepsin in brain tissue as in muscle and two fifths as much as in spleen. They also have worked out a partial purification of this fraction.

Trypsin.—Uber & McLaren (28) have studied the ultraviolet inactivation of crystalline trypsin and find that at 2537 Å and at 2804 Å about sixty quanta of radiation are required to inactivate one molecule of trypsin. The activity was determined as the ability to liberate carboxyl groups from α-benzoyl-*l*-arginine amide.

McDonald & Kunitz (29) have found that crystalline trypsinogen is completely and autocatalytically transformed into trypsin when calcium is present in concentrations greater than 0.02 M. Below this concentration the transformation is complicated by the formation of appreciable amounts of an inert protein which the authors could not convert into trypsin. From studies of the effect of varying the nature

and concentration of salts on the conversion it was found that salts increase and decrease the rate of both reactions so that the final amount of trypsin obtained depends on the nature and concentration of salt used.

Irving, Fruton & Bergmann (30) have obtained first-order hydrolysis constants of crystalline trypsin, papain, ficin, and beef spleen cathepsin on simple synthetic peptides. Hofmann & Bergmann (31) have added benzoyl-glycyl-*l*-arginine amide to those synthetic substrates hydrolyzed by crystalline trypsin. It is of some interest that crystalline trypsin hydrolyzes this peptide ten times faster than benzoyl-arginine amide. Znamenskaya (32) finds that edestin reduced by nascent hydrogen is more susceptible to trypsin digestion than is the original edestin.

Peck (33) reports that potassium soaps inhibit crystalline trypsin. Horwitt (34) finds that insulin reduces protease activity of human and rabbit blood and inhibits the tryptic (Fairchild) action on casein at pH 6.8, but relatively little at pH 8.0. Collier (35) has found that a crude extract of ascaris inhibits pepsin and trypsin but not papain. The trypsin-inhibiting factor has been purified by fractional precipitation in 85 per cent ethanol and by 0.7 saturated ammonium sulfate. It is probably of low molecular weight since it is not precipitated by trichloracetic acid and it dialyzes through cellophane. It combines rapidly with trypsin at pH 7.0.

Ross & Turner (36), in studying the digestion of carbon monoxide hemoglobin by pancreatic enzymes, found that a uniform hemeprotein fragment was not obtained, as shown by the variability in the iron / nitrogen ratio and amino acid content of the acetic acid-insoluble fraction. They also found that the rate of digestion was affected by the state of the heme of the hemoglobin, the rate decreasing in the following order: globin > methemoglobin > oxyhemoglobin > reduced hemoglobin > carbon monoxide hemoglobin.

Huggins & Neal (37) find that prostatic fluid of several animals, including man, contains proteolytic enzymes. One of the enzymes appears to act on fibrin and another on fibrinogen. Semen of dogs and some humans was found to contain small amounts of a proteolytic enzyme which, because of its action at pH 7.5, they call trypsin.

Andersen (38) claims that assays of pancreatic trypsin in duodenal juice of children two weeks to five years of age serve as a reliable means of diagnosis of pancreatic deficiency.

Banner, Dereniuk & Thomas (39) have found that split products

formed by the action of pancreatin or activated papain on insulin have no physiological activity.

A method of estimating trypsin has been reported by Ferguson (40) in which the digestion of fibrin is followed by turbidity estimations in an Evelyn photocolorimeter. The author has also noted the effect of crystalline trypsin on prothrombin (41).

Chymotrypsin.—Brand & Kassel (43) have determined the sulfur amino acids, tyrosine, and tryptophane of chymotrypsinogen, the precursor of chymotrypsin. They find 1.22 per cent methionine and 4.5 per cent "half cystine" which accounts for all of the sulfur. The tyrosine content is 2.96 per cent and the tryptophane 5.51 per cent. This latter analysis is the highest tryptophane content yet recorded for a protein. From these analyses they were able to estimate the molecular weight and obtained a value of 36,700 $\pm$ 100 which speaks well for the purity of the protein and for the methods of analysis. In later papers these authors report an arginine content (44) of 2.83 per cent and a threonine content (45) of 9.4 per cent.

Butler (42) finds the activation energy involved in the conversion of chymotrypsinogen to chymotrypsin by trypsin to be 16,300 calories. Following the increase in formol titration Butler finds 5 $\pm$1 amino groups produced when the precursor is changed into the enzyme. This is in good agreement with the original findings of Kunitz & Northrop (46). Very little, if any, nonprotein nitrogen and no ammonia were liberated in the reaction which indicates that the reaction involves the opening of peptide bonds in ring structures. Butler also finds that deuterium oxide has no apparent effect on the rate of conversion.

As pointed out in the General and Theoretical Section, Fruton & Bergmann (12) have found that chymotrypsin, depending on the substrate used, may behave as an amino peptidase, carbonyl proteinase, and, as originally claimed, an endopeptidase.

Ross & Tracy (47) find that the carbon suboxide (C_2O_3)-treated egg albumin is digested several times faster by chymotrypsin than is native egg albumin. The pH of maximum digestion is the same for the two materials. The total hydrolysis is greater for the native than for the malonylated (carbon suboxide treated) by about ten linkages per molecule. The results of rate studies are not so definite in the case of malonylated serum albumin, but the native serum albumin is split at forty more linkages per molecule than was the malonylated serum albumin.

Pepsin.—The alkali denaturation of pepsin has been taken up again,

this time by Conn, Gregg, Kistiakowsky & Roberts (48) who have followed the calorimetric changes. The difference in heats of reaction of native and denatured pepsin, which they call heat of denaturation, was found to depend on the initial and the final pH of the experiment. The heat of denaturation was found to decrease more rapidly than the enzymatic activity with increasing pH. Between pH 4.3 and 8.2 the heat of denaturation was 16.06 cal. per gm. of nitrogen. This is close to the value previously found by them for methemoglobin (49).

These authors also found that the titration curve of pepsin is altered by making the solution alkaline to pH 9.0. The change consists of an increase in strength or number of five groups per mol of pepsin. This seems rather interesting in the light of Steinhardt's findings (50) that the rate of inactivation of pepsin is a function of the fifth power of the hydrogen ion concentration, i.e., involves five groups having a pK of 6.76.

Herriott (51) has found that when pepsin is treated with weak solutions of iodine at pH 5.0 to 6.0, the iodine goes on to the tyrosine as previously claimed (52) and not as Philpot & Small (53) suggested on to some unknown grouping. Herriott found that under these conditions monoiodotyrosine was first formed in the protein instead of diiodotyrosine. From a preparation of iodinated pepsin which had lost only 10 to 20 per cent of its activity and contained only 0.7 per cent iodine, he was able to account for most of the iodine as monoiodotyrosine. Electrophoresis patterns of the iodinated pepsin were indistinguishable from those of the original pepsin while their solubility curves were very different. Some of the properties of the crystalline monoiodotyrosine were given.

Sprissler (54), in a thorough study, has prepared shark pepsinogen from gastric mucosa by a method paralleling that used for the preparation of crystalline swine pepsinogen (55). He was unable to obtain crystals of the shark protein, but he found that in many properties the precursor from shark was like that from swine. He also studied the pepsin prepared from shark pepsinogen. The specific activity on hemoglobin is the same as swine pepsin, but the milk-clotting activity is only one sixtieth as much.

Treatment of pepsin with carbon suboxide (C_2O_3) which malonylates the protein has been studied by Tracy & Ross (56). They find that the phenolic groups are covered and that there is a loss in activity. On treatment at pH 4.0 for many days there is a partial recovery of activity.

They also found that malonyl serum albumin is digested further

by pepsin than the original serum albumin is by nearly 25 per cent. This is in sharp contrast to their results using chymotrypsin (47). In this case the malonylated derivative was digested less than the original or untreated proteins.

Herriott (57) has described the crystallization of a polypeptide which inhibits the milk-clotting activity of pepsin at pH 5.8. The inhibitor is derived from pepsinogen upon its autocatalytic conversion into pepsin. The reversible combination of pepsin with the inhibitor appears to follow quantitatively the simple mass law. The inhibitor does not retard pepsin at pH 2.0. Certain specie specificity relationships were studied and a few chemical and physical properties examined. The inhibitor appears to be digested by pepsin with a maximum rate near pH 3.5.

Haugaard & Roberts (2), as already mentioned in the General and Theoretical Section, have studied in detail the action of pepsin on lactoglobulin. In addition to what has already been said, they find that whereas the alkali-denatured lactoglobulin is hydrolyzed more rapidly than the native, the extent is greater in the native, being 20.8 per cent of the bonds split against 13.5 per cent in the denatured. These authors also present calorimetric data showing that the heat evolution is not proportional to the extent of hydrolysis. They suggest that a nonhydrolytic but exothermic process accompanies hydrolysis.

Croxatto & Croxatto (58) have found that pepsin acting on any of several proteins produces pressor-active materials. Since similar pressor-active substances are produced by the action of the kidney substance, rennin, they conclude that rennin is a protease. Helmer & Page (59) have confirmed these findings. This subject is discussed in more detail elsewhere in this volume.

Ficin.—Faust & Thomen (60) have found that purified ficin deteriorates rapidly as a parasiticide. Crude latex is relatively stable. Ficin acts on whipworms and hookworms but little, if any, on dogs, ascarids, or tapeworms. Molitor, Mushett & Kuna (61) discuss the toxicology and pharmacological results of the use of ficin with experimental animals.

Irving, Fruton & Bergmann (30) have obtained a first order velocity constant for the hydrolysis of benzoyl-arginine amide by ficin plus cysteine.

Andrews & Cornatzer (62) state that ficin can go through the digestive tract of a dog with most of its activity unchanged. At pH 5.0

ficin digests egg albumin until the amino nitrogen / total nitrogen ratio is 0.25.

Those interested in ficin may care to know of the recent papers by Asenjo & Capella de Fernandez (16) on the papain-like proteinase from fresh maya (*B. pinguin*) which digests live tissue, also the similar proteinase from *Pileus mexicanus* obtained by Castañeda, Gavarron & Balcazar (17) which was found to possess powerful antihelminthic activity.

Rennin.—Schöbel & Rambacher (63) purified rennin by means of the chromatographic technique. They found that the purified product was rapidly inactivated by papain. Those interested in rennet activity may find something of interest in the recent announcement of the crystallization of chymopapain by Jansen & Balls (14) and the findings of Sprissler (54) on shark pepsin, previously discussed. Berridge, Davis, Kon, Kon & Spratling (64) report using rennet from living calves and find that the pH of maximum proteolytic activity is pH 3.0 to 4.0.

Carboxypeptidase.—Dale (65) has studied the effect of x-rays on crystalline carboxypeptidase. He found that the per cent inactivation was a function of the concentration of enzyme for a given dose of radiation. A definite amount of radiation energy absorbed corresponded to a constant amount of enzyme inactivated. Interestingly enough, inactivation did not occur when the substrate was present while the control was 85 per cent inactivated.

Irving, Fruton & Bergmann (66) show that swine kidney carboxypeptidase hydrolyzes carbobenzoxyglycyl-*l*-phenylalanine giving first-order reaction constants in the presence and absence of air. In an atmosphere of nitrogen the rate is double that of air and only half in a one to one mixture of oxygen and nitrogen. The activity is 2.5 times greater when cysteine activation is in nitrogen as in air. They find that only one and the same bond in the above substrate is split regardless of the method of activation. They conclude that they have only one enzyme in their preparation.

Aminopeptidase.—Johnson (67) using brewers' bottom yeast has purified a peptidase which has many of the properties of an aminopeptidase. It hydrolyzes the linkage nearest the amino group, and a carboxyl group is not essential in the substrate. He finds that it splits tripeptides more rapidly than dipeptides and leucyl-glycine much faster than glycyl-glycine. Zinc and halogen ions are necessary for full activity. Johnson obtained a preparation by acetone precipita-

tion which was homogeneous in the ultracentrifuge and by electrophoresis. Using yeast aminopeptidase purified by this method (67), Croxatto & Croxatto (68) find that it rapidly destroys the vasoconstrictor properties of hypertensin and pepsitensin. They also report that purified hypertensinase has some aminopeptidase action.

Maschmann (69) reports that the dipeptidase present in obligate anerobic bacteria is a variable mixture of substrate specific enzymes or a number of apoenzymes with one coenzyme (Fe^{++}). These enzymes are inactive in anerobic conditions and need cysteine and ferrous ion or manganous ion as activators. It is suggested that the mechanism of activation consists of (*a*) a reduction which takes time and (*b*) the binding of ferrous ion. The activated enzyme system splits leucyl-glycyl-glycine and diglycyl-glycine. This system is a mixture as Maschmann demonstrated by separating the bacterial peptidase preparation into two substrate specific enzymes. The enzyme splitting diglycyl-glycine was purified by fractional precipitation with ammonium sulfate.

Schneider & Graef (70) and Schneider (71, 72, 73, 74) have extended the original findings of Grassmann, Volmer & Wündbichler (75) that yeast aminopeptidase, although inactive toward leucyl-glycine, will digest it if a boiled yeast extract is added. A great increase in activity is found if chloride is also added. Schneider & Graef (70) found that histidine has an inhibitory action on yeast aminopeptidase.

Abderhalden (76) points out that results from his earlier experiments on animal peptidases were very much like those of Schneider & Graef (70). Weil & Jennings (77) have found cathepsin and aminopolypeptidase in all structural elements of the rabbit kidney.

Other peptidases.—Maschmann (78, 79, 80) has studied in some detail the peptidase activity of rabbit, guinea pig, and sheep sera and finds that they can be measurably activated by as little as 4×10^{-6} M cobaltous ion or manganous ion and by 1×10^{-4} M magnesium ion. Cysteine, hydrocyanic acid, and pyrophosphate inhibit the peptidase activity, and although the hydrocyanic acid inhibition is not readily reversed, the cysteine inactivation is reversed by manganous ion but not by magnesium ion. The activity of the enzymes was not affected by reagents which react with carbonyl groups. Fractionation by acetone or alcohol resulted in a separation of the serum enzymes into dipeptidases and tripeptidases. In a later paper (81) he was able to report the separation of alanyl-glycine-splitting enzymes from glycyl-glycine-splitting enzymes by again fractionating with acetone.

Gailey & Johnson (82) present evidence for the existence of at least three distinct dipeptidases in swine intestinal mucosa extracts. They also find that peptidases are unstable in water but are stable in 50 per cent glycerin. Most of the peptidase activity was for leucyl peptides.

Abderhalden & Caesar (83) have investigated the occurrence of polypeptidases in the central and peripheral nervous systems.

Holter & Lindahl (84) have studied the distribution of peptidase in *Paracentrolus* embryos.

Nakayama (85) has investigated the hydrolysis of (*o*-, *m*-, or *p*-) glycyl-amino-benzoic acid by peptidases from kidney, spleen, liver, and lung. He found that the *meta*- and *para*- hydrolyze more readily than the *ortho*-. Benzoic acid, aniline, and amino benzoic acid all inhibit the hydrolysis of glycyl-glycine which Nakayama considers explains why the glycyl-amino-benzoic acids are not hydrolyzed to completion.

Smith & Bergmann (86) report that the intestinal enzyme hydrolyzing *l*-leucyl-glycine is a manganese-protein complex and that the combination of metal and protein takes time. Thus at pH 7.9 and 40° C. a solution of 50 μg. of protein nitrogen and 0.01 mM manganous sulfate required four hours to become fully active. They also find that there are other manganese-activatable peptidases in the swine intestinal mucosa extract.

Berger, Johnson & Baumann (87) have studied the relative rates at which *d*- and *l*-peptides are split by peptidases from animal, plant, and bacterial sources. They found that the hydrolysis of the unnatural forms of leucyl-glycine and leucyl-diglycine was, for most peptidases systems, relatively slow. However, some bacterial preparation produced relatively rapid hydrolysis of these unnatural substrates.

Bamann & Schimke (88) present evidence that plant enzymes do not attack the *d*-peptides in a *dl*-mixture. However, later (89) these same authors report that glycerin extracts of human ovaries will split solutions of pure *d*-peptides but only slightly the *d*-component of a *dl*-mixture. Bamann & Schimke (90) have also studied the effect of cobaltous and manganous ions on the activation of dipeptidases.

Matuo (91), by adsorption on kaolin at pH 4.0, was able to separate peptonase and tripeptidases of intestinal juice from dipeptidases and halogen acylases. The dipeptidase was separated from halogen acylase by virtue of the fact that only the latter was destroyed at 60° C.

Matuo (92) also found that the action of dog intestinal juice on peptone was practically unlimited since there were no acid-hydrolyz-

able peptide linkages found after an exhaustive digestion with the juice. This is a little surprising in view of his claim (93) that intestinal juice does not digest proteins like albumin, fibrin, or edestin but does attack casein or gelatin slowly.

Autolytic proteases.—Reisse (94) has continued his line of study and has found that the autolytic processes of muscle tissue of the marine polychete, *Arenicola marina,* at pH 7.5 take place within the oxidation-reduction potential range of —200 to +400 mv. with a maximum at 0.0 mv. The normal potential for living tissue is +87 mv.

Strain (95) finds that autolysis of yeast is not inhibited by oxidizing agents that inhibit the papain type enzyme but is inhibited by hydrogen sulfide.

Synthesis and plastein formation.—The literature on plastein formation is rather controversial. Strain & Linderstrøm-Lang (96) were unable to obtain any evidence for the synthesis of peptide bonds by aeration of papain digests as reported earlier by Maver, Johnson & Voegtlin (97). Strain & Linderstrøm-Lang point out that only in strongly concentrated digests does the nitrogen, soluble in about 12 per cent trichloracetic acid, decrease and this is not accompanied by a decrease in Van Slyke amino nitrogen nor basic nitrogen. To account for the changes in solubility of concentrated digests they suggest that sulfhydryl groups of different fragments may form disulfide linkages producing larger compounds insoluble in trichloracetic acid.

Linderstrøm-Lang & Johansen (98) were also unable to effect the synthesis of protein by oxygenation of a fibrin-papain digest as had been reported by Voegtlin, Maver & Johnson (99) and Maver & Voegtlin (100).

Haddock & Thomas (101) report a plastein insoluble in 3.3 per cent trichloracetic acid formed by the action of Difco trypsin on a concentrated peptic digest of insulin. A similar product was obtained by the action of papain on a concentrated papain digest of insulin and a third product by the action of papain on a concentrated peptic digest of insulin.

Flosdorf (102) warns that antigenicity is not evidence in favor of the possible protein nature of plastein without careful regard for the specificity of the antigen.

Gol'dshtein & Tsiperovich (103) believe they obtained plastein from the action of papain on peptone at pH 4.7. The precipitate which was obtained over a period of five hours was insoluble in

water and was hydrolyzed by pepsin. It had a lower amino-nitrogen content than the original peptone and gave a much higher biuret test. Hydrogen peroxide inhibited the enzyme formation of plastein.

Reviews and Books

Several articles have appeared reviewing particular phases of the work. Thus, Bergmann & Fruton (104), Bergmann (105), and Fruton (106) review their work. Johnson & Berger (107) have covered the subject of peptidases. Van Slyke (108) has reviewed the subject of kinetics of hydrolytic enzymes and their bearing on methods for measuring enzyme activity.

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THE STEROIDS

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The progress in the biochemistry of the steroid hormones (1), vitamins (2), and glycosides (3) has been reviewed individually in preceding volumes of this series. The present chapter deals primarily with the biochemistry of the oldest members of the steroids, namely, the bile acids (4) and the sterols (5), and also with chemical reactions leading from these compounds of which there is an abundant supply, to substances of pharmaceutical significance, which are less accessible in nature.

Naturally Occurring Bile Acids

Table I summarizes the bile acids found in the biles of numerous species of animals which have been analyzed since 1936 when we gave a complete list of the bile acids identified at that time [(6), p. 22 ff.]. The assiduous research by Japanese workers failed to yield novel results except in a few of the lower vertebrates. Chenodesoxycholic acid (= anthropodesoxycholic acid = 3,7-dihydroxycholanic acid) frequently has been found to accompany cholic acid whose general occurrence in mammals, birds, and fish has been repeatedly confirmed. Anthropodesoxycholic acid has been isolated from pig bile by Ido (16) who in turn confirmed the occurrence of 3-hydroxy-6-ketoallocholanic acid, previously isolated by Fernholz (44), Sugiyama (15), and by Anchel & Schoenheimer (14). A stone from a hog's gall bladder contained lithocholic acid (17), first found in ox gallstones and later in minute amounts in human bile. Ursodesoxycholic acid, previously found in bear bile conjugated with taurine, has now also been isolated as a glyco-conjugate (10). Reduction of 3-hydroxy-7-ketocholanic acid yielded ursodesoxycholic acid in small amounts besides chenodesoxycholic acid (11). Sapocholic acid, one of the weaker bile acids of ox bile, has been formulated $C_{30}H_{48}O_3$ by Wieland & Seibert (18); a monoacetyl derivative indicates the presence of a hydroxyl group; the acid may be pentacyclic and not a steroid proper. α-Lagodesoxycholic acid, considered by its discoverer Kishi (45) as the C-12 epimer of desoxycholic acid, has to be attributed

different steric configuration, as Koechlin & Reichstein (46) find a different melting point and rotation for the authentic 12-epidesoxycholic acid obtained from 3-hydroxy-12-ketocholanic acid.

An instance of a nonconjugated bile acid, whose natural occurrence has been observed in abnormal and probably in normal human bile [see (6), p. 29 ff.], has been found in chicken bile from which Maeda (22) isolated free chenodesoxycholic acid. Taurochenodesoxy-

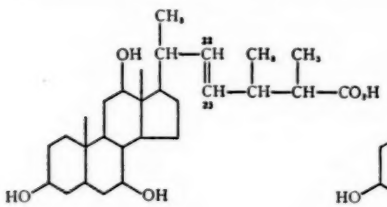
TABLE I

Species	Acid	Conjugation
MAMMALS		
Primates		
Monkey (*Pithecus cyclops* (7)	Cholic, desoxycholic, anthropodesoxycholic	Glycine
Carnivora		
Leopard (10)	Cholic	Taurine
Fox (9)	Cholic, desoxycholic	Glycine
Bear (10)	Ursodesoxycholic	Glycine, taurine
Polar Bear (12)	Cholic, β-phocaecholic (no α-phocaecholic)	
Raccoon (13)	Cholic, desoxycholic	
Marten (13)	Cholic, desoxycholic	
Weasel (9)	Cholic	
Fishotter (9)	Cholic, desoxycholic	
Ungulata		
Hog (14)	3-Hydroxy-6-ketoallocholanic	
Hog (16)	Anthropodesoxycholic, α- and β-hyodesoxycholic, 3-hydroxy-6-ketoallocholanic	
Hog [gallstone (17)]	Lithocholic	
Cattle (18)	Sapocholic ($C_{30}H_{48}O_3$)	
Rodentia		
Rabbit (19)	Cholic	
Guinea pig (20)	Chenodesoxycholic, 3-hydroxy-7-ketocholanic	
Marsupialia		
Kangaroo (8)	Cholic, desoxycholic, chenodesoxycholic	Glycine
BIRDS		
Domestic duck (21)	Chenodesoxycholic, cholic	
Wild duck (21)	Chenodesoxycholic, cholic	
Turkey (21)	Chenodesoxycholic, cholic	
Fowl (22)	Chenodesoxycholic	Unpaired
Fowl (23)	Chenodesoxycholic	Taurine
Pheasant (24)	Chenodesoxycholic	Taurine

Species	Acid	Conjugation
REPTILIA		
Turtles [*Emys orbicularis* (25), *Amyda japonica* (26)]	Trihydroxysterocholanic lactone	
Snakes [*Elaphe quadrivirgata, Bungarus multitinctus* (27)]	Cholic	Taurine
AMPHIBIA		
Toad [*Rana catesbina* (28)]	Tetrahydroxycholane, trihydroxybisnorsterocholanic	
Toad (29)	Pentahydroxybufostane, tetrahydroxycholane	
FISH		
Teleostei		
Salmo ["Masu" (30)], *Stereolepis* ["Ishinaga" (37)], *Mugil* ["Hirami" (35)], *Thynnus thynnus* ["Maguro" (37)]	Cholic	Taurine
Sardine ["Iwashi" (31)], *Silurus*, sheet fish, *Pararulus* (32), *Conger myriaster* ["Maanago" (32)], *Theragra cholcogramma* ["Suketo" (34)], *Paralichthys olivaceus* (34), *Sparus macrocephalus* (36), *Euthynnus pelamis* (38), *Nibea mitsukurri* (39), *Siganus fuscescens* (30), *Sebastodes matsubarae* ["Ako" (40)], *Sebastodes inermis* ["Mebaru" (41)], *Monacunthus cirrhifer* ["Kawahagi" (41)]	Cholic, chenodesoxycholic	Taurine
Pelteobagrus nudiceps, recte Pimelodus fulvidracus ["Gigi" (33)]	Cholic, tetrahydroxynorsterocholanic	Taurine
Elasmobranchii		
Blue skate (42)	Scymnol	
Grey dogfish (42)	Scymnol	
Shark (43)	Cholic, acids $C_{27}H_{46}O_6$ or $C_{28}H_{48}O_6$	

cholic acid from chicken bile has been dehydrated (24) to taurochenoapodesoxycholic acid which, in turn, upon hydrolysis and further dehydration led to a choladienic acid; the latter on hydrogenation yielded β-apocholic acid; this suggests that the original removal of a hydroxyl group on C-7 has been followed by a shift of the double bond to position 8:14.

The lactone of a tetrahydroxysterocholanic acid, $C_{28}H_{40}O_5$, has been isolated from the bile of several species of turtle and has been characterized by its triformyl ester and the corresponding tetraketo acid (25). This lactone is isomeric with the trihydroxybufosterocholenic acid (I) (47). The two isomeric forms of the latter, both found in toad bile by Shimizu *et al.* (47), have been converted into isomeric bufosterocholanic acids to which has been ascribed the carbon skeleton of ergosterol. Shimizu & Kazuno (48) attribute the isomerism to the attachment of the carboxyl group on the one-but-last and second-but-last carbon atom, respectively, of the side chain. Pentahydroxybufostane (3,7,12,24,25-pentahydroxyergostane) (II) has been isolated from toad bile where it occurs as a sulfuric acid ester, and a number of its chemical transformations have been described (29).



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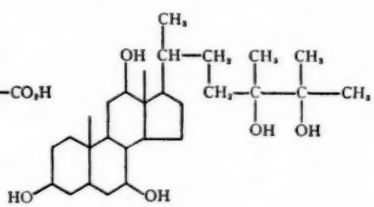
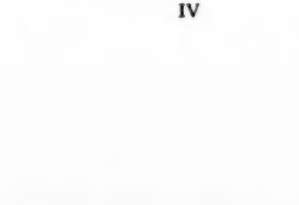


II

III



IV

Another fraction of the same bile contains "tetrahydroxycholestane" which yields on oxidation with chromic acid 3,7,12-triketo-20,22-epoxycholane. Another tetrahydroxycholestane occurs as a sulfuric acid ester which is dehydrated during hydrolysis to trihydroxycholene. The acid fraction of the same bile contains trihydroxybisnorsterocholanic acid, $C_{26}H_{44}O_5$ (28).

In the bile of the Gigi fish 3,6,12,24-tetrahydroxysterocholanic acid (III), $C_{27}H_{46}O_6$, was found (33). Oxidation of the side chain, after partial acetylation in the 3 and 6 positions, led to the corresponding diacetate of 12-keto-3,6-dihydroxycholanic acid (IV) which was converted by the Wolff-Kishner reduction method to hyodesoxycholic acid (49).

Bile Acid Metabolism

The metabolism of natural bile acids and their derivatives has been studied, again mostly by the Japanese, in the living animal and under the influence of microorganisms. The value of many papers on this subject is impaired by the known difficulties encountered in the differential analysis of bile acids and by the lack of purity of the bile acids used. The alleged formation of the tricyclic desoxybilianic acid from dehydrodesoxycholic acid in the toad (50) and the formation of desoxycholic acid from 12-keto-Δ^3-cholenic acid in the rabbit (51) may be explained by impurities of the respective starting materials. Perfusion experiments on the surviving liver with potential precursors of bile acids suffer from the difficulty of collecting sufficient material for analysis. If one eliminates from consideration all such doubtful observations, there still remain a number of examples for the reduction of keto groups to hydroxy groups. One of the three keto groups in dehydrocholic acid, namely, that in position 7, is reduced in the rabbit (52) to yield 7-hydroxy-3,12-diketocholanic acid. 3-Keto-7,12-dihdyroxycholanic acid is converted by the toad upon subcutaneous injection into the C-3-epimer of cholic acid (53), found in unconjugated form in the urine. Similarly, dehydrodesoxycholic acid is reduced to 3-hydroxy-12-ketocholanic acid, which is presumably a partial racemate, i.e., a mixture of the two C-3-epimers (54). Sasaki (55) claims that reductodehydrocholic acid (3-hydroxy-7,12-diketocholanic acid) is reduced in the organism of the guinea pig to desoxycholic acid whose conjugate with glycine is found in the gall bladder of the experimental animals but not in the normal controls. This observation implies complete reduction of the keto group at C-7

which is also claimed to be reduced in the case of dehydrocholic acid in the rabbit organism (56). The much debated conversion of cholic acid into desoxycholic acid, involving complete reduction of the hydroxyl group at C-7, has again been reported from observations on guinea pig liver pulp (55) and in the putrefaction of the pancreas (57) [see also (58) p. 371]. *B. coli communis* (59) and *B. proteus* (60) reduce specifically the carbonyl group on C-7 of dehydrocholic acid. *B. coli communis* also reduces both keto groups of dehydrochenodesoxycholic acid (61). "β-Dehydrohyodesoxycholic acid" (3,6-diketoallocholanic acid) is reduced in the rabbit and 3(β), 6(α)-dihydroxyallocholanic acid is excreted in the urine (62). The labile "α-dehydrohyodesoxycholic acid" (3,6-diketocholanic acid) is allegedly converted into 3(α), 6(α)-dihydroxyallocholanic acid by the rabbit (63).

Another example of isomeric rearrangement is the formation of dihydroxycholenic acid from apocholic acid in the toad (64). The studies, reviewed above, exemplify biological hydrogenations of keto acids and throw little light on the catabolism of the natural bile acids. However, in two recent communications on cholic-acid metabolism (65), Schmidt & Hughes demonstrate the disappearance of injected cholic acid in the guinea pig, confirming and explaining older observations of the negative bile-acid balance in the animal organism. Intravenously injected cholic acid appears in the gall bladder [cf. observation in man (66)]; it does not reach the large intestine but vanishes in the ileum due to the action of the microorganism *Alcaligenes fecalis* under aerobic conditions (65). A quantitative study by means of a modified Gregory & Pascoe method for cholic acid determination (67) and of Hughes gravimetric mercuric-iodide-hydrazone method for keto acids (68), indicates stepwise oxidation of the three hydroxyl groups (that on C-3 being least reactive) to dehydrocholic acid as end product. The very low cholic acid content of the liver itself, an incidental finding in these studies, throws an interesting light on the nature of bile-acid formation. Similar experiments on gall bladder fistula dogs illustrate the increase of ketocholanic acids in the bile above their normal level upon administration of natural bile salts and keto bile salts (69).

The pathways of oxidation of the C_{28}- and C_{27}-steroids from the bile of amphibia and of elasmobranch fish are discussed by Kazuno (29). The occurrence of pentahydroxybufostane, scymnol (hexahydroxycoprostane), tetrahydroxynorbufostane, tetrahydroxynorsterocholanic acid, and trihydroxybufosterocholenic acid in bile sug-

gests, according to this author, that oxidation from ergosterol and 7-dehydrocholesterol to C_{24}-bile acids follows two mechanisms: oxidation of terminal methyl groups of the side chain, followed by β-oxidation on the one hand; and oxidation of double bonds *via* epoxyderivatives to glycols, on the other hand.

Conjugated Steroids

The conjugation of steroids with amino acids and other hydrophilic radicals, whether performed by nature or by the synthetic chemist, aims at enhancing the physiological activity by increasing the solubility in water. The production of taurocholic acid in the dog, upon administration of sulfur-containing amino acids and related derivatives, has been studied by Virtue & Doster-Virtue (70). Frankel (71) confirmed, over an extended pH range, the prevailing assumption that liver tissue in contrast to renal histozyme is unable to hydrolyse conjugated bile acids. He isolated microorganisms from human and animal intestines and feces, which could hydrolyse conjugated bile acids and could use them as their sole source of carbon and nitrogen. Cortese (72) has continued his studies on *in vitro* conjugation of bile acids. By an analogous procedure, dicholylcysteine and, from it, cholylcysteic acid have been prepared (73). Attempts to conjugate cholic acid with the N^4-atom of sulfanilamide were designed to impart cholotrophic character to the sulfa drugs (74). Hydrazides of bile acids have been shown to form crystalline derivatives with numerous carbonyl compounds (75).

Choleic Acids

The list of choleic acids, the peculiar coordination compounds of desoxycholic acid and of apocholic acid [(58) p. 112 ff., p. 121], has been augmented by a series of compounds whose acholic components are long chain paraffines and related substances (76). Undecane forms crystalline complexes with six molecules of both desoxycholic or apocholic acid. Pentadecane, hexadecane, pentatriacontane ($C_{35}H_{72}$), and tritetracontane ($C_{43}H_{88}$) all combine with the bile acid component in the molecular ratio 1 : 8. The same ratio obtains for the unsaturated hydrocarbons, cetene and Δ^{18}-heptacosene, and also for the mixed aromatic-aliphatic cetylbenzene and *p*-hydroxyphenyloctadecane. The choleic acid of vitamin K was prepared with a view to purification of concentrates of this vitamin (77). A pale yellow fraction, m.p. 185°, showed considerable vitamin K activity. The complex contains about

10 per cent of the acholic component, which indicates a molecular ratio of vitamin to acid of 1:8. Less conclusive is the proof, based on spectrographic data, of the existence in alcohol-carbontetrachloride solution of a coordination compound of β-carotene with desoxycholic acid in equimolecular proportions (78). Caglioti & Giacomello (79) applied Patterson-Harker analysis to the study of the molecular lattices of choleic acids, especially of palmitic acid–choleic acid. Their findings confirm the previously obtained x-ray data on the cell dimensions of choleic acid molecules and the prevailing conceptions of the steric constellation of the ring system in bile acids (cf. p. 70).

Physical Properties of Bile Acids

The dipole moment of ten hydroxy- and ketocholanic acids, measured in dioxane, has been found to vary from 2.50, for lithocholic acid, to 5.63, for dehydrocholic acid (80). The absence of abnormality in the dipole moments of desoxycholic and apocholic acids speaks against the existence in dioxane solution of dioxane-choleic acid which is known to exist in the crystalline state.

The effect of bile salts and of soaps on the hydrophobic side chains of proteins has been suggested as explanation for the effects of these compounds on the "structural" viscosity of plant protoplasm (81). Interesting observations on the denaturation of protein by bile acids (82) and on the preparation and colloidal properties of protein sols containing bile salts (83) have been reported. Gel formation observed in solutions of desoxycholic acid in phosphate buffers of various pH values (84) is presumably due to interaction of desoxycholic acid with the phospate buffer (85). The properties of monomolecular layers of cholanic acid were investigated by Sobotka & Bloch (86) who found that the presence of one hydroxyl group, as in lithocholic acid, interferes with the formation of stable films and the presence of a second hydroxyl group prevents film formation altogether.

Older data by Josephson (87), who worked with aqueous solutions, on the dissociation constants of bile acids have been revised by Kumler & Halverstadt (88). These workers used about 0.01 *N* solutions in 50 per cent alcohol and extrapolated their values to pure aqueous solutions. The dissociation constants are, in terms of pK units, from 0.3 to 1.4 units higher than Josephson's values. Bile acids may be considered as γ-substituted valeric acids and the dissociation constants, revised on the basis of these new observations, actually range between 1.02 and 1.12×10^{-5} for the natural mono-, di-,

and trihydroxycholanic acids. The value for dehydrocholic acid is 1.24×10^{-5} as compared to 1.32×10^{-5} for caproic acid; it seems but plausible that these constants should fall into a narrow range in view of the remoteness of the modifying functional groups from the carboxyl group.

Analysis of Bile Acids and Sterols

An improvement of the Gregory & Pascoe procedure for the determination of cholic acid is suggested by the use of acetic acid instead of ethanol to overcome turbidity [(89) cf. (67)]. The green fluorescence emitted by the products obtained with a mixture of acetic and sulfuric acids has been applied to the measurement of the bile-acid content of blood, urine, and other materials (90). Only a slight trace, less than the minimum detectible amount of 0.25 mg. per 100 ml., is found by this method in normal serum or whole blood which yields values up to 20 mg. per 100 ml. in jaundice. Normal urine excreted during a twenty-four-hour period contains from 6 to 16 mgs. of cholic acid.

An old modification of the Pettenkofer reaction using vanillin and concentrated phosphoric acid has been revived (91) for the colorimetric determination of cholic acid and of cholic acid plus desoxycholic acid. A spectrophotometric evaluation of the furfural-phosphoric acid reaction was also reported (92). Still another method, derived from the Pettenkofer reaction for the evaluation of cholic acid, is the carmine color developed with levulose and concentrated hydrochloric acid at 70° (93). A color reaction, using as reagent benzaldehyde and 75 per cent sulfuric acid, is recommended as a qualitative test for desoxycholic acid. The free acid gives a color change from red to green, while conjugated desoxycholic acid gives a blue color. Some other bile acids produce a purple color (94). The same authors (95) describe a color reaction with *m*-dinitrobenzene for 3-hydroxy-12-ketocholanic acid and 3-hydroxy-7,12-diketocholanic acid, supplementing the Zimmerman reaction for 3-keto bile acids and other 3-ketosteroids. Several dehydration products formed in the course of the Hammersten-Yamasaki reaction of cholic acid with hydrochloric acid were studied in order to elucidate its mechanism (96).

Modifications of the Gregory & Pascoe reaction for cholic acid, a colorimetric iron method for total bile acids, the Schmidt and Dart method for conjugated bile acids, sulfur determination for taurine, and polarimetric measurements in various combinations were used with

advantage in the differential analysis of normal and pathological gall bladder bile (97, 98). Composition of human gall bladder bile was studied in its relations to calculus formation by Reinhold and coworkers (99).

The color reactions of bile acids, sterols, and steroid hormones [either alone or in the presence of furfural (100) or benzaldehyde (101)] upon layering with concentrated sulfuric acid have been studied extensively with special consideration of the structural and steric position of functional groups.

Other analytical developments in the field of bile acids include the isolation and quantitative determination of natural and experimentally produced keto acids by means of Girard's reagent (68), of hydroxylamine (69), and of *p*-carboxyphenylhydrazine (14); the preparative separation of bile acids in cattle bile has been improved by a method of fractional extraction of the ethereal solution with hydrochloric acid (18).

The action of pyridinium sulfur trioxide upon cholesterol yields the pyridonium salt of cholesteryl sulfate which could be converted into other salts (102). Cholesteryl sulfate, ergosteryl sulfate, and other steryl sulfates may be used for the analytical and preparative separation of these sterols from natural sources and from reaction mixtures.

The application of chromatographic methods to the separation and analysis of sterols, bile acids, steroid aglycones, vitamins, and hormones has been summarized with many practical details (103).

Phytosterols

The search for new steroids as a potential starting material for the partial synthesis of sex hormones has stimulated continued investigations of phytosterols.

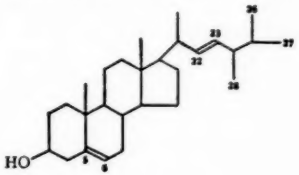
Phanerosterols.—Both the C_{29}- and the C_{28}- orders are well represented among the phanerosterols, the sterols of higher plants.

(*a*) C_{29}-Order.—Incompletely purified and characterized sterols of the sitosterol type have been obtained from *Typha* (104), from *"Semen coicis,"*[1] the latter presumably identical with α-sitosterol (105). Phytosterolins, i.e., sterol glycosides, from soy bean and cottonseed oil have been studied by Thornton, Kraybill, *et al.* (106) who

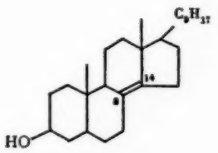
[1] We are unable to verify this name and are giving it as it appears in *Chem. Abstracts* (105).

identified stigmasterol as one of the aglucones and *d*-glucose as the carbohydrate component.

(*b*) C_{28}-Order.—The doubly unsaturated brassicasterol of rape seed oil, hitherto considered a C_{29}-sterol, has to be reformulated with twenty-eight carbon atoms according to Fernholz & Stavely (107). The saturated tetrahydrobrassicasterol (brassicastanol) is identical with ergostanol and ozonization of the side chain of brassicasteryl acetate yields methylisopropylacetaldehyde plus acetoxy*bis*norcholenic acid; hence the configuration of an ergostadienol-$\Delta^{5:6,22:23}$ has been ascribed to brassicasterol (V). The isomeric doubly unsaturated α-spinasterol is formulated by Fernholz & Ruigh (108) as $\Delta^{8:14,22:23}$-ergostadienol (VI). Bessisterol, a sterol from the root of *Momordica*



V



VI

cochinchinensis, was also identified with spinasterol (109). In addition to both the above ergostadienols, Fernholz (110) elucidated the structure of a new mono-unsaturated C_{28}-sterol of m.p. 157–158° and $[\alpha]_D = -33°$, first isolated from *Brassica campestris* and named "campesterol." It also occurs in soy bean and wheat germ oil, but not in tall oil and cottonseed oil. Perbenzoic acid titration showed the presence of one double bond. Gentle chromic acid oxidation of the saturated campestanyl acetate yielded 3(β)-hydroxynorallocholanic acid. An *i*-methyl ether could be obtained from the campesteryl-*p*-toluene sulfonate, indicating the presence of a double bond between C-5 and C-6 (see p. 70). Energetic chromic oxidation led to a dextrorotatory methylisoheptylketone (dihydrothujaketone) $CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(CH_3) \cdot CH(CH_3)_2$, $[\alpha]_D = +12°$, whereas ergostenol and other ergosterol derivatives give the levorotatory isomer, $[\alpha]_D = -14°$ to $-16°$. This elegant proof shows that campesterol is enantiomeric on C-24 with $\Delta^{5:6}$-ergostenol (111).

Other unsaponifiable fractions of higher plant extracts are of rather doubtful nature. Acetone extracts of plantation rubber contain, in addition to β-sitosterol, small amounts of a sterol formulated by Heilbron *et al.* (112) as a stigmastenol with the double bond presum-

ably between C-24 and C-28. A substance $C_{30}H_{49}OH$, designated as caoutchicol, has also been isolated from crude rubber extracts (113); its nonprecipitability with digitonin casts doubt on its steroid character. Another euphorbiacea, *Euphorbia lathyris,* contains in its seeds 0.6 per cent of a crystallizable substance of m.p. 199°, $[\alpha]_D = +170°$, designated euphorbiosteroid, but the formula $C_{29}H_{36}O_7$ practically excludes its formulation as a steroid (114). Finally, a substance $C_{20}H_{28}O_3$ was found in the oil from coffee beans, accompanied by γ-sitosterol. Because of its alleged estrogenic activity a steroid formula with a short side chain of two or one carbon atoms was ascribed to it (115, 116, 117), but Wettstein & Miescher (118) adduce ample evidence that it is not a steroid compound.

Cryptosterols, algal sterols.—A valuable summary of algal sterols will be found in Heilbron's review of algal chemistry (119). The typical algal sterol, fucosterol, formulated by Heilbron as $\Delta^{5:6,14:15}$-sitosterol is specific for *Fucus* and all other Phaeophyceae, Bacillariophyceae, and Chrysophyceae. Chlorophyceae and Rhodophyceae, in many respects closer related to the higher plants, contain with few exceptions sitosterol, the characteristic sterol of the phanerogams. The primitive Myxophyceae are devoid of sterols. This distribution of the steroids among the various classes of algae is paralleled by the alternating occurrence of xanthophyll in the higher classes and fucoxanthin in the lower ones, while myxoxanthophyll is confined to the primitive Myxophyceae. Exceptions from this distribution of steroids and of carotenoids dovetail with taxonomic and ecological deviations amongst some algal species. The unexplained correlation of sterols and carotenoids with each other in algae finds an equally puzzling counterpart in the simultaneous deposition of cholesterol and carotenoids in vertebrate animals under physiological and especially pathological conditions.

Fungisterols.—The conditions under which yeast produces a maximum of sterols have been studied from numerous angles; aerobic conditions are most favorable and the formation of sterols, greatest in the early stages of fermentation, runs parallel with that of other lipoids (120). Zymosterol found in ergosterol residues from yeast was identified as $\Delta^{8:14,24:25}$-cholestadienol and numerous of its derivatives were identified with certain α-cholestenol derivatives (121). This sterol shares with spinasterol the distinction that none of its double bonds is situated between C-5 and C-6. The formula of another yeast sterol, cryptosterol (an ambiguous designation since it is also used for crypto-

gam sterols in general), is still uncertain according to Wieland (122) who prepared many new derivatives without deciding on a final structural formula.

Zoosterols

Sterols of invertebrates.—Sterols of lower animals, especially of the marine fauna, display a much greater variety than those of the higher animals. W. Bergmann (123) has reinvestigated many sterols from invertebrate sources in the light of the newer knowledge of the steroids. In many instances the character of the sterols found pointed to their exogenous alimentary origin, e.g., the presence of cholesterol in the carnivorous sea anemones, the presence of phytosterol-like sterols of the order C_{29} in herbivorous bivalves as the oyster.

Cnidaria.—Corals such as *Madrepora cervicornis, Meandra areolata,* and *Gorgonia* contain in addition to hydrocarbons, ketones, waxes (cetyl palmitate), and cetyl alcohol, a mixture of sterols comprising cholesterol and an unknown sterol $C_{29}H_{50}O$, perhaps identical with clionasterol (123).

Porifera.—The old "clionasterol" from sponges such as *Cliona celata* and *Spheciospongia vesperia* was recognized as a mixture of a mono- and a di-unsaturated sterol (124). The term clionasterol has been retained for the former ($C_{29}H_{49}OH$ rather than the old formula $C_{27}H_{45}OH$). The di-unsaturated sterol $C_{29}H_{47}OH$, designated as poriferasterol, is hydrogenated to a saturated sterol closely resembling ostreastanol (124, 125). From the fresh-water sponges, *Spongilla lacustris* and *Ephydatia fluviatilis,* a C_{29}-sterol has been isolated (126) which contains one double bond whose position is still controversial (125, 127, cf. 230).

Vermes and Mollusca.—The antirhachitic provitamin fraction of the earthworm *Lumbricus terrestris* and of *Arion empiricorum* and of the snail *Helix pomatia* has been identified with ergosterol while that of the snail *Buccinum undatum* is 7-dehydrocholesterol (128).

Arthropoda.—Sterols obtained from insects and insect eggs appear to be related to sitosterols and are presumably of alimentary origin (129).

Vertebrates.—The α- and β-epimers of 7-hydroxycholesterol have been found in nature, the former in ox liver (130) and in hog liver (131) and the latter in pregnant mare serum (132). The β-form was found to be identical (132) with the product previously obtained by Heilbron and co-workers (133) through oxidation of cholesteryl

hydrogen phthalate with permanganate. No other new sterols have been discovered in vertebrate materials. Phytosterols of alimentary origin occur in the feces of sheep and fowl (129). Cholesterol itself has been identified in anterior pituitary extracts (134).

The occurrence of cholesterol in the urine is apparently not a specific symptom of individuals afflicted with malignant tumors, but rather specific for diseases like nephritis and typhoid fever (135).

Chemical Reactions of Steroids

Three problems stand out in the partial synthesis of steroid hormones and of vitamin D from sterols or bile acids: (*a*) oxidative degradation of the side chain; (*b*) aromatization of ring I; and (*c*) introduction of hydroxyl groups into position 7 of ring II and position 11 of ring III.

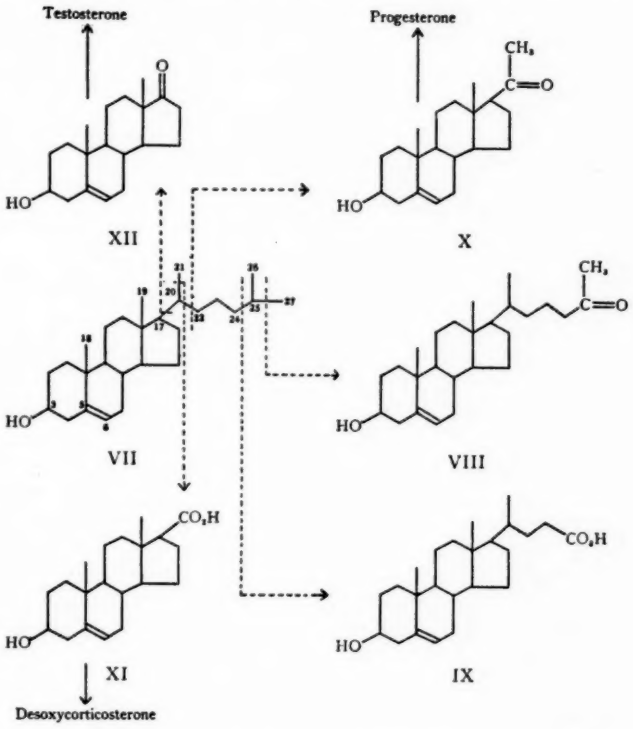
Oxidative degradation of the side chain.—The oxidative degradation of the side chain by chromic acid or ozonization has been widely used in the exploration of the structure of sterols and bile acids. On the one hand, these oxidations yield typical aliphatic fragments [e.g. (111)]; on the other hand, they yield acidic and neutral compounds containing a tetracyclic nucleus. Ruzicka first applied these procedures to preparative ends, namely, to the production of steroid hormones.

Oxidation of cholesterol (VII) with chromic acid yields a great variety of fragments: elimination of a methyl group in the side chain results in the formation of ketones (VIII) still retaining the main chain. The next point of least resistance is between C-24 and C-25 and cleavage at this point gives 3(β)-hydroxy-$\Delta^{5:6}$-cholenic acid (IX). Cleavage at C-20 yields 3(β)-hydroxy-$\Delta^{5:6}$-etiocholenic acid (XI), and $\Delta^{5:6}$-pregnenol-3-one-20 (X); the complete elimination of the side chain yields $\Delta^{5:6}$-androstenol-3-one-17 (XII) ("dehydroisoandrosterone"). Among these products, pregnenolone and dehydroandrosterone are intermediates for the commercial preparation of progesterone and testosterone; the etiolithocholenic acid is the starting material for the synthesis of desoxycorticosterone.[2]

Several methods have been described for obtaining progesterone directly from cholesterol dibromide. Spielman & Meyer (136) used acid permanganate and obtained yields of about 0.2 per cent of crude

[2] We are indebted to Dr. Erwin Schwenk for permission to reproduce the included diagram.

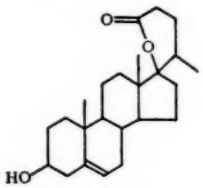
material besides about 50 per cent of cholestenone which could be further oxidized to progesterone by the chromic acid method of Dirscherl & Hanusch (137). By blowing oxygen through melted cholestenone in the presence of vanadium pentoxide at 170° Bretschneider (138) obtained progesterone. Marker and co-workers (139) oxidized



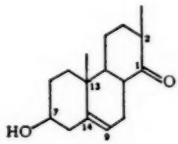
cholestene dibromide-5,6 with chromic acid and obtained $\Delta^{5:6}$-androstenone-17 which they transformed into "desoxo"-testosterone and testosterone.

Ruzicka & Fischer (140) further studied the products obtained on chromic acid oxidation of cholesterol acetate dibromide and isolated nor-(—27)-$\Delta^{5:6}$-cholestenol-3-one-25 (VIII) and $\Delta^{5:6}$-pregnenol-3-one-20 (X). A lactone (XIII) was isolated by Miescher & Fischer

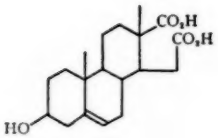
(141) from the oxidation products of cholesterol, while Koester & Logemann (142) identified a phenanthrene derivative (XIV) formed by opening of ring IV. Hydrogenation of the double bond led to 7-hydroxy-2,13-dimethyl-perhydrophenanthrone-1, identical with the product obtained from β-ergostenol acetate peroxide (143). Ring IV is also broken in the tricyclic 3-hydroxy-$\Delta^{5:6}$-etiobilienic acid (XV) found by Kuwada (144) among the oxidation products of dibromocholesterol acetate.



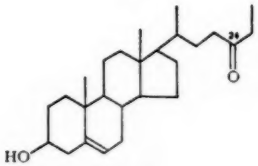
XIII



XIV



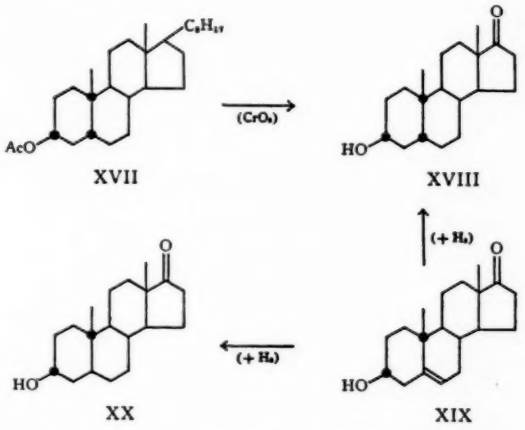
XV



XVI

From the oxidation products of sitosterol acetate dibromide, Mitui (145) isolated dehydroisoandrosterone (XII), 3-hydroxy-$\Delta^{5:6}$-norcholenic acid 3-hydroxy-$\Delta^{5:6}$-etiobilienic acid (XV), and 3-hydroxy-$\Delta^{5:6}$-nor-cholestenone-24 (XVI). The latter substance was synthesized by the action of ethyl magnesium bromide on the amide of acid (IX). Sitostanyl acetate and stigmastanyl acetate similarly yielded androstanol-3(β)-one-17, 3-hydroxynorallocholanic acid and 3-hydroxyetioallobilianic acid (146). Campestanol on oxidation with chromic acid yielded 3(β)-hydroxynorallocholanic acid (111), previously obtained by a similar treatment of ergostenol. From the ozonization of brassicasterol acetate, 3-acetoxybisnorcholenic acid was obtained (107). Reichstein (147) obtained etiocholanol-(β)-one-17 (XVIII) from the chromic acid oxidation of coprosterol (coprostanol) acetate (XVII). The same product resulted from hydrogenation of

$\Delta^{5:6}$-androstenol-3(β)-one-17 acetate (XIX) together with the isomeric etioallocholanol-3(β)-one-17 (androstanolone) (XX). This result definitely establishes the steric position of the hydroxyl group in coprostanol (XVII).



XVII XVIII XX XIX

Marker & Wittle (148) describe a new method for degrading ketones of the pregnane series to acids with 20 carbon atoms; the ketones condensed readily with benzaldehyde and the condensation product was oxidized with chromic acid to the etiocholanic acid. Another method for the degradation of pregnane derivatives consists in oxidation with potassium persulfate; 3(β)-hydroxyallopregnanone-20 yielded 3(β)-hydroxyetioallocholanic acid and a mixture of carbinols from which androstandione was obtained on chromic acid oxidation (149).

The pyrolytic degradation of the side chain of cholesteryl chloride was used by Bloch & Rittenberg (150) in their investigation of the fate of deuterium, introduced by the animal organism into cholesterol. Besides isooctane and isooctene they obtained a hydrocarbon $C_{19}H_{30}$.

Direct oxidation of the side chain of bile acids fails to yield well-defined products. Hence, the esters are converted by the Grignard reaction into tertiary carbinols which are dehydrated to ethylenes; these, in turn, are oxidized to the next lower homologue of the original acid [(58), p. 591]. This so-called Barbier-Wieland degradation is repeated stepwise. Sawlewicz & Reichstein (151) extended and improved the degradation of lithocholic acid, first described by Reindel &

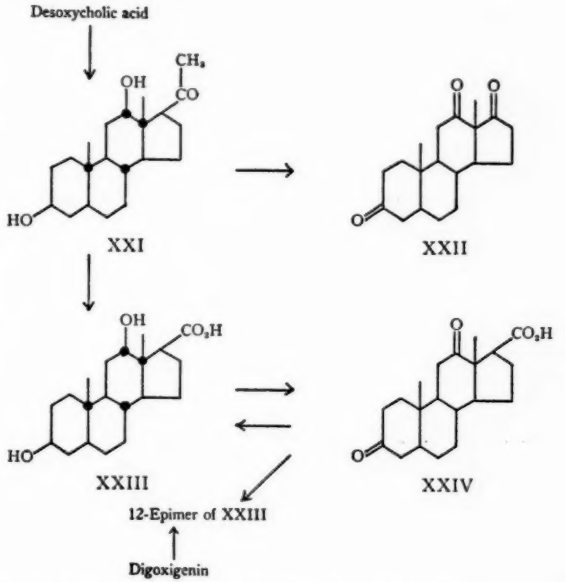
Niederlaender (152), and obtained etiolithocholic acid. Hoehn & Mason (153) performed a similar degradation and obtained pregnanol-3(α)-one-20 and etiolithocholic acid; they transformed the latter into 3-ketoetiocholanic acid and 4-bromo-3-ketoetiocholanic acid. Since natural lithocholic acid is difficult to isolate, the material is usually prepared from desoxycholic acid or from cholic acid *via* the 3-hydroxy-12-keto- or 3-hydroxy-7,12-diketo-acid and Wolff-Kishner reduction (154, 155, 156).

Steiger and Reichstein (155, 157) similarly degraded $\Delta^{5:6}$-hydroxy-*bis*norcholenic acid (obtained by oxidation of stigmasterol) to 3(β)-hydroxy-$\Delta^{5:6}$-etiocholenic acid, the starting material for their synthesis of desoxycorticosterone. This acid is more readily obtainable from the acidic oxidation of cholesterol acetate dibromide (cf. p. 60) and is now the most suitable material indeed for the preparation of etiolithocholic acid and etioallolithocholic acid by reduction of the intermediate 3-keto-$\Delta^{4:5}$-etiocholenic acid (158).

The degradation of desoxycholic acid has been described by Mason & Hoehn (159) who obtained 3,12-dihydroxyetiocholanylmethyl ketone (XXI) which by oxidation yields 3,12,17-triketoetiocholane (XXII) and etiodesoxycholic acid (XXIII). The latter was transformed into dehydroetiodesoxycholic acid (XXIV); catalytic reduction of this diketo acid yielded a mixture of isomers from which 12-epi-etiodesoxycholic acid was isolated which proved to be identical with the dihydroxyetiocholanic acid obtained by Steiger & Reichstein (160) in the degradation of digoxigenin. Reichstein & von Arx (161) describe a similar degradation of desoxycholic acid *via* the *bis*noracid first described by Sawlewicz (162). They prepared a number of 3,12,20-hydroxy- and ketopregnane derivatives and suggest that the 3,12,17-triketoetiocholane (XXII) of Mason & Hoehn (159) actually contains the pregnane skeleton [cf. (163)]. They (161) also obtained the 3,12-dihydroxyetiocholanic acid (XXIII) but did not succeed in degrading it to the dihydroxyetiocholanone. Improved methods for preparing pure desoxycholic acid from commercial material (164) and from cholic acid (165) have been described.

Kimura & Sugiyama (166) performed the degradation of hyodesoxycholic acid to 3,6-dihydroxypregnanone-20, which was independently obtained by Marker & Krueger (167) who transformed it into progesterone. Chenodesoxycholic acid was likewise subjected to treatment by the Wieland-Barbier method and yielded 3,7-dihydroxyetiocholanic acid and 3,7-dihydroxypregnanone-20 (168). Morsman,

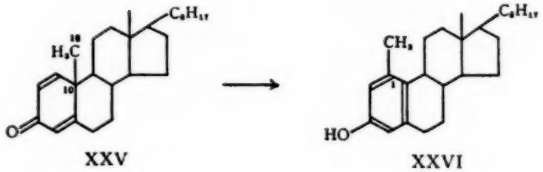
Steiger & Reichstein (169) first obtained 3,7,12-trihydroxypregnanone-20 by degradation of cholic acid. Their work was repeated by Ehrenstein & Stevens (170), who also succeeded in isolating etiocholic acid and transformed the 3,7,12-trihydroxypregnanone-20 into 7,12-dihydroxyprogesterone.



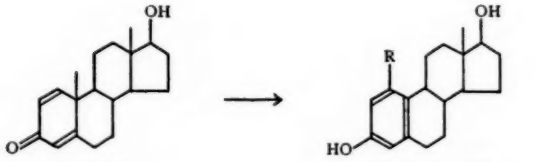
The elimination of the terminal carboxylic carbon atom of cholic acid (171) and of desoxycholic acid (172) has been accomplished by Curtius degradation yielding amines with 23 carbon atoms.

Aromatization of ring I.—The vast amount of research aiming at transformation of cholesterol into estradiol [cf. (5), pp. 148 ff.] has finally been successful (173, 174). $\Delta^{1:2,4:5}$-cholestadienone-3 (XXV) was isomerized by acetic anhydride and sulfuric acid into a phenol (XXVI) of m.p. 145–146°, with migration of the angular methyl group C-18 from C-10 to C-1. The aromatic character of this compound was proved by coupling with benzene diazonium chloride to a red azo dye. The ultraviolet absorption spectrum was reasonably close to that of estradiol. By a similar reaction $\Delta^{1:2,4:5}$-androstadienol-17-one-3 (XXVII) yielded 1-methylestradiol (XXVIII) which was

without estrogenic activity. Estradiol (XXIX) itself was obtained in small yields by pyrolysis at a temperature of 325° of the same androstadienolone, the angular methyl group being eliminated as methane (174).



XXV XXVI

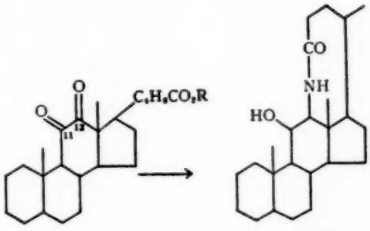


XXVII XXVIII. R = CH_3
XXIX. R = H

Introduction of hydroxyl groups and double bonds.—Fortunately, the oxygen function on C-3 in the three groups of sex hormones is preformed in cholesterol and need only be protected by acetylation during the side chain degradation discussed above. Less convenient for the synthetic chemist is the position of the hydroxyl group on C-11 in corticosterone and the position of the double bond in ring II between C-7 and C-8 in the provitamins D.

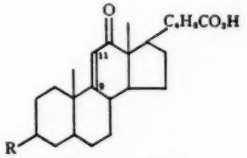
Various attempts have been made to introduce an oxygen function into position 11 of the steroid molecule. Such compounds would pave the way for the synthesis of a number of cortical hormones, especially corticosterone. No successful method has been described so far, but a number of interesting observations have been reported. Desoxycholic acid is used as starting material since presence of the hydroxyl group at position 12 greatly facilitates further transformation of ring III. Barnett & Reichstein (175) prepared 12-ketocholanic acid by a modification of the procedure of Wieland. This compound was transformed into a mixture of α- and β-11-hydroxy-12-ketocholanic acids and oxidized to 11,12-diketocholanic acid (XXX). Wolff-Kishner reduction of the ester did not yield the expected

hydroxy-acid, but the hydroxylactam (XXXI). Alther & Reichstein (176) improved the method of preparation of 12-hydroxycholanic acid and prepared from it by thermic dehydration $\Delta^{11:12}$-cholenic acid. Hydroxylation with osmium tetroxide yielded 11,12-dihydroxycholanic acid which, on chromic acid oxidation, yields an 11,12-dicarboxylic acid (*neo*lithobilianic acid); this proved to be identical with material prepared by oxidation of 11-hydroxy-12-ketocholanic acid. A similar sequence of reactions led from 3-keto-$\Delta^{11:12}$-cholenic acid (177) by Meerwein reduction to α- and β-3-hydroxy-$\Delta^{11:12}$-cholenic acid, hence to 3-hydroxy-11,12-epoxycholanic acid. This compound when reduced catalytically yielded only lithocholic acid and desoxycholic acid (178) and not the desired 3,11-dihydroxycholanic acid. Bergstrøm (179) reduced 12-keto-$\Delta^{9:11}$- cholenic acid (XXXII) by the Wolff-Kishner method and obtained apparently $\Delta^{9:11}$-cholenic acid. Bergstrøm & Haslewood (156) prepared a monosemicarbazone from 11,12-diketocholanic acid which on hydrolysis yielded 11-hydroxy-12-ketocholanic acid. Wolff-Kishner reduction of the semicarbazone gave a triazine of m.p. 292–295°. Marker & Lawson (180) prepared 3-hydroxy-12-ketocholanic acid by the procedure of Kaziro & Shimada (181) and transformed it into 3,11-dihydroxy-12-ketocholanic acid. The same acid was prepared by Longwell & Wintersteiner (182) in slightly higher yield and purity. Attempts to reduce the keto group with hydrazine and sodium ethylate yielded a compound of m.p. 262°



XXX

XXXI

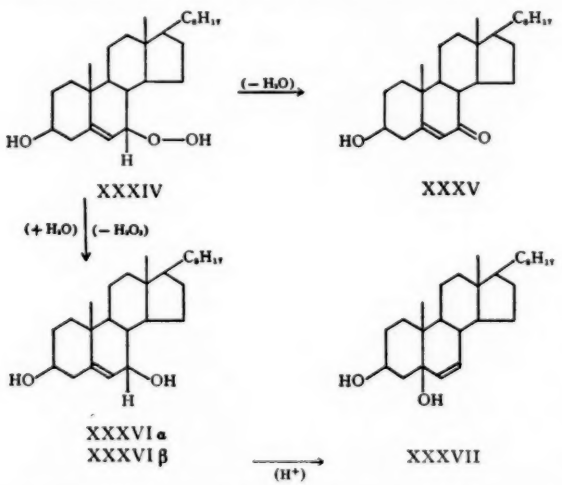


XXXII. R = H
XXXIII. R = OH

which seemed to contain only 3 oxygen atoms. On repeating this work, Marker and co-workers (183) isolated a 3,11,12-trihydroxycholanic acid of m.p. 136°. Chakravorty & Wallis (184) also started from 3-hydroxy-12-ketocholanic acid. This was brominated and the bromo acid treated with sodium ethylate to yield 3-hydroxy-12-keto-$\Delta^{9:11}$-

cholenic acid (XXXIII). Wolff-Kishner reduction gave an acid of m.p. 183–184°, to which the authors ascribe the structure of 3(α)-hydroxy-$\Delta^{9:11}$-cholenic acid.

A new method for the introduction of a hydroxyl group on C-7 has been described: treatment of cholesterol in aqueous colloidal solution with oxygen led to a mixture of oxidation products characterized by the color reaction of Lifschütz (sulfuric plus glacial acetic acid). Wintersteiner & Bergstrøm (185) investigated this reaction in detail and succeeded in isolating a number of products. The primary reaction products are 7-ketocholesterol (XXXV) and α- and β-7-hydroxycholesterol (XXXVI) presumably formed *via* a peroxide (XXXIV). Of these, the α-7-hydroxycholesterol was isolated from the nonketonic part of the reaction mixture. The β-isomer seems to undergo, under the influence of acids, an allylic rearrangement to $\Delta^{6:7}$-cholestenediol-3,5 (XXXVII). 7-Ketocholestadiene was isolated



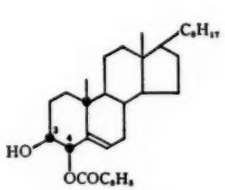
in small amounts as a secondary product. The speed of reaction depended on the temperature; at 85° about 40 per cent ketone and 20 per cent diols were formed within a few hours; at 37° the reaction proceeded much slower. Cyanide, phenol, hemin, manganese, etc., inhibited the reaction. The cyanide-inhibited system could be reactivated by cupric ion. A minimum amount of heavy metal is probably essential for the progress of the reaction. Other steroids of cholesterol type

(stigmasterol, campesterol, fucosterol, 3(β)-hydroxy-$\Delta^{5:6}$-etiocholenic acid) showed essentially the same behavior on oxidation. Esterification of cholesterol greatly diminished the susceptibility towards oxygen. The facile oxidation of cholesterol under almost physiological conditions is especially interesting because of the natural occurrence of some of the oxidation products (130, 131, 132). Although secondary formation of these oxidation products during isolation cannot be excluded, Wintersteiner (132) considers it probable that they are intermediates in the physiological synthesis of provitamin D_3.

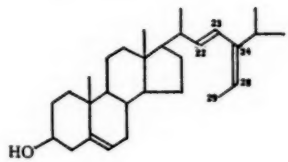
Improvements have also been made in the dehydration of 7-hydroxycholesterol to $\Delta^{5:6,7:8}$-cholestadienol-3 (provitamin D_3). Rosenberg (186) substituted treatment of the dibenzoate of 7-hydroxycholesterol with boiling dimethylaniline for the older method of pyrolysis. Recently, Wintersteiner & Ruigh (187) suggested use of the 7-monobenzoate; the free cholestadienol could be easily separated from unreacted material and by-products as the digitonide, since 7-(α)-benzoxycholesterol is not precipitated by digitonin. A similar, unexpectedly soluble digitonide has been reported for 3-hydroxy-4-*cis*-benzoxy-$\Delta^{5:6}$-cholestene (XXXVIII) (188).

Further studies on the introduction of hydroxyl groups and formation of double bonds in ring I or II have been reported by Petrow & Starling (189), Spring & Swain (190), Jackson & Jones (191), Pirrone & Vannucchi (192), Sah (193), and Hattori (194). The use of various reagents such as copper sulfate, quinoline, and collidine (195) on halogenated steroids has been studied with special regard to the position of the resulting double bonds. A number of cholestadienes and cholestenes have been compared (196) and the shifting of double bonds in them studied (197). The two pairs of bromine atoms in tetrabromo-stigmasterol display different degrees of reactivity as the halogens in position 5:6 may be removed with sodium iodide (199). The stabler bromine atoms in the side chain can be removed as such by alcoholic potassium hydroxide or aromatic bases with the recovery of stigmasterol, whereas potassium acetate at high temperatures removes two molecules of hydrogen bromide, yielding stigmastatrienol (XXXIX) (200). The catalytic hydrogenation with platinum oxide in aqueous-ethereal solution of 5,6-dihalogen derivatives of cholesterol and other sterols was studied by Decombe & Rabinowitch (201), who showed that cholesterol dibromide regenerated cholesterol while cholesterol dichloride formed 6-chlorocholestanol, which could be further hydrogenated to cholestanol. The action of perbenzoic acid on choles-

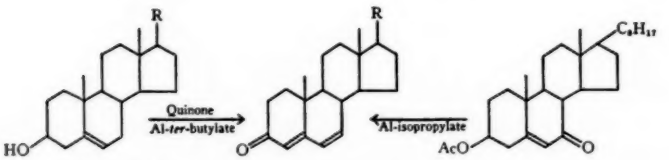
tadiene-2 : 3,4 : 5 did not yield a transannular oxide, as in the case of ergosterol, but cholestene-2 : 3-diol-4,5 (202). Potassium cholesteryl sulfate is decomposed on heating with sodium *sec.*-octylate with the formation of cholestadiene-3 : 4,5 : 6 [(203), cf. (102)]. An interesting reaction is the simultaneous dehydrogenation of the hydroxyl group on C-3 and of ring II by a modified Oppenauer reaction using benzoquinone as hydrogen acceptor. This makes possible the facile preparation of 6-dehydrotestosterone benzoate (XLI) from the 17-monobenzoate of androstenediol-3,17 (XL) and the preparation of 6-dehydroprogesterone (XLIII) from pregnenol-3-one-20 (XLII) (204). The formation of $\Delta^{4:5,6:7}$-cholestadienone-3 (XLV) as by-product in the Oppenauer dehydrogenation of 7-ketocholesteryl acetate (XLIV) is also noteworthy (205). The application of the conventional Oppenauer reaction in the preparation of cholestenone is described in *Organic Syntheses* (206).



XXXVIII



XXXIX



XL. R = $OCOC_6H_5$
XLII. R = $COCH_3$

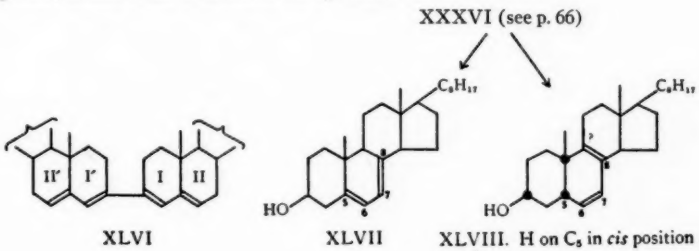
XLI. R = $OCOC_6H_5$
XLIII. R = $COCH_3$
XLV. R = C_8H_{17}

XLIV

Photochemical reactions.—The photodehydrogenation of ergosterol (207, 208), cholestadiene-2,4 (208), cholestadiene-5,7 (209) and the photoisomerization of several α,β-unsaturated ketones of the cholestenone type (210) under the influence of ultraviolet light have been studied. In the case of α,β-unsaturated ketones (210), pinacols are also formed; these are not changed by further irradiation but when a

chloroform solution of the pinacol from cholestenone is exposed to sunlight, water splits off, probably under the influence of hydrochloric acid formed from the solvent. The resulting hydrocarbon $C_{54}H_{86}$ shows ultraviolet absorption at 313 mμ indicating the presence of four conjugated double bonds (XLVI). The mutual transformation of apocholic acid and the isomeric dihydroxycholenic acid under the influence of ultraviolet light (211) may also be due to the presence of hydrochloric acid formed during irradiation, since these two acids are known to be in equilibrium in the presence of hydrochloric acid.

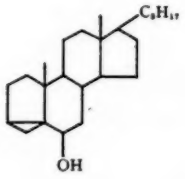
Zuehlsdorff (212) studied the ultraviolet irradiation products of $\Delta^{6,8}$-cholestadienol-3 (XLVIII) obtained as a by-product in the preparation of 7-dehydrocholesterol, i.e., $\Delta^{5:6,7:8}$-cholestadienol-3 (XLVII). The first step consists of isomerization at C-5, yielding $\Delta^{6,8}$-coprostadienol (XLIX); no antirhachitic properties developed on further irradiation but the carbon skeleton was probably changed, since no Diels' hydrocarbon was obtained by selenium dehydrogenation. A steric rearrangement also takes place during ultraviolet irradiation of estrone (213). The new product (lumi-estrone) on dehydrogenation with palladium black yielded *l*-isoequilenin, identical with the synthetic product of Bachmann, Cole & Wilds (214), while estrone by similar dehydrogenation treatment yielded *d*-isoequilenin which is identical (214) with the 14-*epi*equilenin of Hirschmann & Wintersteiner (215). Since these two epimeric isoequilenins differ only in their steric arrangement at carbon atom C-13, the isomerization of estrone on ultraviolet irradiation must be due to rearrangement at this point; hence lumi-estrone is 13-*epi*estrone.



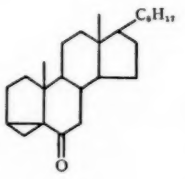
Isocholesterol.—Several puzzling observations on supernumerary isomers in the sterol field have found a satisfactory explanation (216). The so-called *i*-ethers of cholesterol are now formulated as ethers of

i-cholesterol with a three-membered ring instead of the double bond and with the hydroxyl group on C-6 (L). Heterocholestenone, previously considered to be 6-ketocholestene-3 : 4, was found to be identical with *i*-cholestanone (LI) (217) obtained by oxidation of *i*-cholesterol. The interrelation of these substances and some of their derivatives and oxidation products are summarized by Ladenburg *et al.* (218). The same type of isomerization explains the peculiar "transesterification" of 3-monoesters of $\Delta^{5:6}$-cholestenediol-(*cis*)-3,4 to the respective 4-monoesters (XXXVIII) (219). The occurrence in urine of labile "pentacyclic" isomers of the androstane and pregnane series is suggested by Rosenheim (219) and may account for the formation of 3-chloro derivatives during hydrolysis with hydrochloric acid in the usual isolation procedure. This isomerization has been utilized for the protection of the cyclic double bond of stigmasterol and brassicasterol during catalytic hydrogenation of the double bond in the side chain (220). The same type of isomerization has been used (221) to protect the double bond in 3(β)-hydroxy-$\Delta^{5:6}$-cholenic acid during Wieland-Barbier degradation of the side chain (cf. p. 55, for *i*-campesterol).

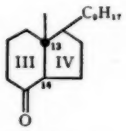
Stereochemistry.—Other investigations of stereochemical interest include the epimerization of the hydroxyl on C-3 by heating with aluminum isopropylate in neutral solution (222) and the study of *syn*- and *anti*oximes of cholestenone (223). The *"trans"* constellation at the juncture of rings III and IV (C-13, C-14) has been confirmed in the ergosterol series: the bicyclic ketone $C_{19}H_{32}O$ (LII), resulting



L



LI



LII

from cleavage of vitamin D_2 between C-9 and C-8, belongs to the *"trans"* series as indicated by its rearrangement under the influence of acids or alkalis into the lower melting and more soluble *cis*-hydrindane derivative (224). Glucosides of epimeric steroids were investigated by Miescher *et al.* (225) who first gained the impression that glucoside formation was confined to the digitonin-precipitable epimers. Subsequently these views were revised (226) when Gillespie (227) and

Linstead (228) demonstrated glucoside formation of both epimers of hydroaromatic and of steroid alcohols. In the instance of cholestanol and epicholestanol, both the α- and β-glucosides were prepared (228). Work along these lines has served as a model in the synthesis of water-soluble hormones and vitamins [e.g. (229)].

The relationship between optical rotation and constitution of the steroids has been studied by Bernstein *et al.* (230) on the basis of recent additions to the experimental data. These authors stress the negligible interaction between asymmetric groups (unless they are in close proximity to each other) in a relatively rigid and flat structure, such as the perhydrocyclopentenophenanthrene nucleus of the steroids. Thus, using cholestane as the substance of reference, fairly constant additive increments to the molecular optical rotation may be computed for functional groups, double bonds, and variations in the length and structure of the side chain. Values calculated on the basis of these results agree well with the experimental data for practically all substances where comparable data are available and permit certain predictions to be made with regard to structure or optical rotation.

Woodward (231) has studied the influence of substituents upon the characteristic ultraviolet absorption band of α,β-unsaturated ketones. Ketones of the type $-CO-CR_\alpha=CR_\beta R_\beta{}^1$, with a single substituent on one of the two ethylenic carbons, showed a peak at 230 mμ or below. All ketones with two substituents either in the α,β- or β,β-positions exhibit selective absorption in the region 239 ± 5 mμ. When no hydrogen is attached to the α and β carbon atoms, the absorption maximum is shifted further, to the region between 249–258 mμ. This rule permits wide application in the field of steroids and has been used in the revision of certain structural formulas (232).

Miscellaneous.—Introduction of other elements into the cholesterol molecule has been achieved. In the case of sulfur, thiocholesterol (233) and thiodihydrocholesterol (234) have been prepared. The mercuration of cholesterol (235) led to 6-chloromercuricholesterol which is transformed by reaction with iodine, in chloroform solution, into the very stable 6-iodocholesterol.

Although the chemistry of lanosterol, a constituent of lanolin, resembles in many respects that of the sterols (236), it has a pentacyclic nucleus and should be placed in the class of triterpenes.

Reference should be made to attempts at synthesis in the field of the cardiac aglycones. Lactones of the digitaligenin type have been synthesized which differ from the natural prototypes only by the absence of tertiary hydroxyl groups (237, 238).

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS

SECTION I

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Topics discussed in this section include the amino acids, peptides, the carbohydrate-containing proteins, and denaturation. It is not to be expected in so complex a subject that spectacular advances can be recorded annually, but steady progress has been made during the year in bringing the chemistry of proteins into line with that of simpler compounds. In addition the virus investigator is now working at the same bench as the protein chemist, so that the links between the study of living organisms and pure chemistry are rapidly becoming stronger.

Analysis.—The evaluation of a number of theories of protein structure depends on accurate amino acid analyses, which in their turn depend upon improvement in technical methods. The Bergmann & Niemann (1) theory of the periodic recurrence of a simple arrangement of amino acid residues in the protein molecule, for example, is dependent on accurate analysis. In the case of edestin Chibnall (2) concludes that newer analyses do not support the periodicity hypothesis and suggests that the reason lies in the fact that the protein molecule is composed of several peptide chains of different amino acid composition. In such a case the composition of the whole protein molecule could not be expected to possess a periodic structure even if the individual peptide chains possessed such a pattern of amino acid arrangement.

Silk fibroin has been found by Meyer (3) not to possess the amino acid composition demanded by a simple periodic structure. Hydrolytic products of silk fibroin with molecular weights between 600 and 1000 have been found by Landsteiner (4) to inhibit the reaction between silk fibroin and its corresponding precipitating serum. Hence it would seem that the structures determining the antigenic behaviour of the protein are no larger than peptides containing only eight to twelve amino acids. It remains to be seen whether the determinant peptides constitute a recurrent pattern in

the silk fibroin molecule or whether they are of the nature of isolated prosthetic groups.

The study of partial breakdown products is obviously of paramount importance in determining the detailed structure of the protein molecule, but this study has not as yet received much attention. An examination of the peptides liberated by the action of cold hydrochloric acid on wool and edestin has yielded promising results, electrodialysis being used to aid the separation of the products. On the average the breakdown products were tripeptides but all of the cystine was in the form of the free amino acid and the basic fractions were mainly dipeptides. The variety of amino acids linked to the dibasic amino acids contradicts the postulate of a simple periodic structure in the native protein (5).

The situation has been greatly clarified concerning the composition of the phosphopeptones obtained by digestion of casein. Amongst earlier workers considerable differences of opinion existed. Recent work on casein phosphopeptones containing eight, nine, and ten amino acids has given closely similar results in the hands of different workers. In a decapeptide Damodaran & Ramachandran (6) characterised three glutamic acid, three isoleucine and four serine residues. Lowndes, Macara & Plimmer (7) found two glutamic acid, two serine, three isoleucine, one aspartic acid, and two phosphoric acid residues in an octapeptide, with the probable addition of a serine, glutamic acid, and phosphoric acid residue in a decapeptide, whilst Rimington (8) found five glutamic acid, four serine, and three phosphoric acid residues in a nonapeptide with the addition of an isoleucine residue in the decapeptide, the phosphoric acid residue being attached to serine.

The chemical composition of the bactericidal polypeptides, gramicidin and tyrocidine, has been partly elucidated (9 to 13). Both contain considerable amounts of *d*-amino acids, gramicidin having 45 per cent and tyrocidine 20 per cent of *d*-α-amino acids. Gramicidin has no free amino or carboxyl groups, but contains 37.3 per cent of *l*-tryptophane, some *d*-leucine, and some hydroxyamino acids; it may have the composition $C_{74}H_{105}O_{13}N_{15}$. Tyrocidine contains tryptophane, tyrosine, alanine, phenylalanine, dicarboxylic amino acids, ammonia, and nitrogen bases; the suggested composition is $C_{63}H_{103}O_{13}N_{13}$. Measurement of the ammonia liberated by the action of alkaline periodate has been utilised for the determination of hydroxylysine, after separation of serine, threonine, and β-hydroxy-

glutamic acid by crystallisation of the phosphotungstates (14). It is noteworthy that Van Slyke, using this method, found appreciable amounts of hydroxylysine only in gelatin and collagen.

Vickery (15) has determined histidine with 3,4-dichlorobenzene sulphonic acid. The results obtained indicate that human, horse, and sheep haemoglobin contain 35, 33, and 32 molecules, respectively, of histidine per molecule of protein and that edestin contains 2.6 per cent of histidine, this figure being subject to correction.

The use of copper salts for the separation of the dicarboxylate fraction of amino acids in the hydrolysate from gliadin led to the isolation of 40 per cent of the protein in the form of glutamic acid, one twentieth part of which was probably in the racemic form (16). Aspartic acid (0.43 per cent of the dry protein) and serine (0.18 per cent) were the only other amino acids present in the fraction precipitated by baryta and ethanol.

Among the analytical results obtained in the case of chymotrypsinogen (17) was an exceptionally high value for tryptophane (5.51 per cent), a zero value for free thiol groups despite the presence of cysteine, and a figure for the minimum molecular weight of 36,700; this is in agreement with osmotic pressure measurements.

The cystine content of tobacco mosaic virus has been reported (18), and 68 per cent of the total protein has now been accounted for analytically (19). Knight & Lauffer (20) were able to differentiate two strains of tobacco mosaic virus by their different amino acid content although they were indistinguishable in the electrophoresis apparatus.

The oxytocic and pressor hormones of the posterior pituitary lobe were found to have a totally different amino acid composition (21). Both hormones had high cystine contents, and it is perhaps significant that reduction of thiol groups in a protein obtained from the pars neuralis of ox pituitary almost completely abolished the physiological activity (22). In the case of insulin, however, the disappearance of activity when the thiol groups are reduced is ascribed to the concomitant denaturation and not merely to the condition of the thiol groups (23).

Studies have been reported of amino acids of rubber latex (24), the protein of *Hevea brasilensis* (25), peanut proteins (26), and the sulphur amino acids of grasses (27).

Crystalbumin resembles insulin and the oxytocic-pressor hormone of the posterior pituitary gland and differs from most other proteins

in containing no methionine (28). It is of interest that crystalbumin differs from many other proteins also in the complete absence of carbohydrate and in its low tryptophane content which corresponds to only one amino acid residue in the protein molecule. The different amino acid compositions of the albumin and globulin fractions of the serum proteins have been described (29).

The application of chromatographic methods to amino acid separations presents interesting possibilities which have not yet been fully explored. Turba (30) has investigated the adsorption behaviour of monobasic amino acids, whilst Martin & Synge (31) have evolved a chromatographic method of separating the higher monoamino acids by preparing the acetyl derivatives, dissolving them in chloroform containing butyl alcohol and allowing the solution to percolate through a silica gel column with methyl orange as indicator. A continuous observation device such as the Toepler Schlieren method is utilized. Tiselius (32) has followed the adsorption of various amino acids and peptides on charcoal. Separation of the constituents is effected by their retardation figures. Tryptophane was retarded greatly and glycine very little; peptides were intermediate in their behaviour, whilst casein and egg albumin were retarded less than peptides. A trypsin digest of casein exhibited the numerous peaks to be expected from its complex composition.

The carboxyl groups in free amino acids have been determined gasometrically and titrimetrically by measuring the carbon dioxide liberated by the action of ninhydrin (33, 34).

Among methods for determining basic amino acids may be mentioned colorimetric methods for arginine and histidine using α-naphthol and diazobenzene sulphonic acid respectively (35), and a micro method for arginine (36). Aromatic sulphonic acids have been used for the determination of various amino acids (37), and Town (38) has used the 3,5-dinitrobenzoyl derivatives to separate the isomers of leucine and valine.

The alcoholic thymol reaction for indican has been applied (39) to the colorimetric determination of tryptophane after preliminary deamination with nitrous acid.

Brief reference may be made to the determination of methionine (40) and a polarographic method for determining cystine (41).

The formation of N-acetyl derivatives of amino acids is suppressed, whilst the acetylation of hydroxyl groups is catalysed by the presence of perchloric acid (42, 43, 44). This has led to the develop-

ment of a titrimetric method for the determination of hydroxyl, —NH, =NH and, with difficulty, sulphydryl groups. Cystine is exceptional in reducing perchloric acid. It is suggested that acetylation of the appropriate amino acid in the presence of perchloric acid leads to the formation of S-acetylcysteine, the indole-nitrogen acetyl derivative of tryptophane, and the imino-nitrogen acetyl derivative of arginine.

Commenting upon the ultraviolet absorption spectrum of papain, Darby (45) emphasises the importance of taking proper account of the general absorption of the protein preparation in using absorption spectra for detecting the presence of tyrosine. It is confirmed that when appropriate precautions are taken the occurrence of amino acids such as tyrosine in the protein molecule results in the appearance of characteristic absorption bands in the absorption spectrum of the protein.

A number of investigations have been reported on the determination and behaviour of thiol groups in proteins (46, 47, 48).

Hydrolysis of proteins.—Lanthionine, $COOH \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, has been isolated, characterised, and synthesised (49 to 54). It is obtained when a number of proteins are heated with alkali before being hydrolysed with acid in the usual way, and results from the action of alkali on the cystine in the protein. This should serve as a useful warning that the isolation of an amino acid from a protein hydrolysate does not necessarily indicate its presence in the native protein, and the possibility must not be overlooked of the formation of artefacts by the action of the somewhat drastic hydrolytic agents usually employed. Racemisation of the amino acids of zein by continued heating with sulphuric acid after the amino acids had been liberated has been reported (55), and serine and threonine have been found (56) to be more stable to alkali when present as free amino acids than when they are combined in the protein molecule. The formation of ammonia during alkaline hydrolysis is frequently in excess of that accounted for by amide groups and arginine (57). Baryta is found to be a more efficient agent for the hydrolysis of egg albumin than sodium hydroxide (58).

In cystine determinations hydriodic acid is found to be the best hydrolytic agent since its use leads to least humin formation, but a mixture of hydrochloric and formic acids gives closely similar results (59).

In searching for tryptophane-containing hydrolysates of proteins

suitable for intravenous injection it has been found (60) that a six hour hydrolysis with 2.6*N* H_2SO_4 of casein and pumpkin seed globulin yields hydrolysates containing 85 per cent and 65 per cent, respectively, of the original tryptophane content of the proteins.

Properties of amino acids.—The acylation of amino acids by 3,5-dinitrobenzoyl chloride and other acid chlorides has been described (61).

Polarimetric measurements indicate that the effect of formaldehyde on proline is to yield a single complex composed of one molecule of each reactant (62). Other polarimetric studies include examination of the rotatory powers of histidine (63) and leucine (64).

The dissociation constants of hydroxylysine obtained from titration curves lend support to the view that the hydroxyl group is attached in the γ or ε position (65). Thermodynamic ionisation constants of hydroxyamino acids and proline (66) and the isoelectric points of threonine and related compounds (67) have been reported.

Observations have been made on the effect of phosphates and of hydrogen ion concentration on the reducing activity of the phenolic group of tyrosine (68).

Iodination of pepsin resulted in the tyrosine being iodinated. Mono- and diiodotyrosine were formed, the former being isolated in a crystalline condition (69).

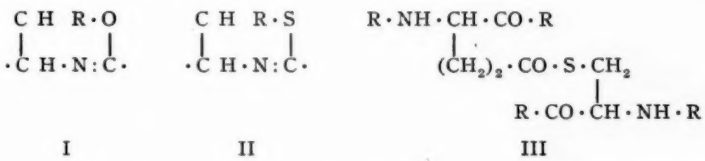
Studies have been reported (70) of the infrared absorption spectra of amino acids and proteins.

Sulphur-containing amino acids and denaturation.—Continuing their studies on cystine in wool, Phillips and his collaborators (71) find that about one third of the cystine in wool reacts with sulphite, the remaining two thirds being resistant. This is attributed not to part of the cystine being in a different condition but to the fact that when some of the cystine —S — S— linkages are ruptured, the stresses on the remaining linkages are lessened and they are less reactive. This is correlated with the supercontraction of hair after treatment with bisulphite and suggests that these disulphide linkages may be responsible for maintaining keratin in the α-configuration.

Almost one third of the total disulphide-sulphur of wool also reacts with formaldehyde at 70° and pH 5.6 (72), and this is probably the same fraction that reacts with bisulphite. Half the disulphide-sulphur that disappears on treatment with formaldehyde is probably converted into combined thiazolidine-4-carboxylic acid.

The effect of methylating agents (73, 74) on wool is to produce

O- and N-methylation. The O-methylation consists partly of esterification of free carboxyl groups but apparently peptide linkages also react. This latter methylation occurs, it is suggested, only at activated peptide linkages, the activation being effected by condensation with the side chains of β-hydroxy acids, such as serine and threonine, giving oxazoline rings (I).



I II III

In reduced wool similar condensation may occur with free cysteine side chains giving thiazoline rings (II), a possibility previously suggested by Linderstrøm-Lang & Jacobsen (75). It is pointed out (74) that the formation of thiazoline rings would mask thiol groups, and that the appearance of free thiol groups when cysteine-containing proteins are denatured may be due to the inhibition of thiazoline ring formation by the denaturing agent.

Interesting suggestions concerning denaturation are made by Bailey (76) and Chibnall (2). Protein molecules, in particular edestin, are regarded as being composed of a number of peptide chains held together by cross-linkages such as the disulphide group and thiol-ester linkages (III). During denaturation such thiol-ester linkages might be ruptured and give rise to free thiol groups. It is suggested that denaturation consists first of depolymerisation into peptide chains which then coalesce in a haphazard manner giving a fibrous structure which may be oriented to resemble the β-keratin structure. In this connection it is of interest that Lundgren (77) also reports the formation of fibres from nonfibrous native proteins by dissociation and denaturation, involving a preliminary unfolding of the chain to give a labile α-form, which in the presence of detergents form fibres. The possibility of unfolding the structure of a protein and remoulding it in a new form has been envisaged by Pauling & Campbell (78) who claim that it is possible to prepare antibodies *in vitro* by subjecting a mixture of the antigen and normal serum globulin to partial denaturation. The formation of homologous antibody is ascribed to an unfolding of the peptide chains of the globulin during denaturation and a

refolding in a configuration complementary to the antigen. If confirmed this observation may prove to be of considerable importance.

The disaggregation of proteins into a small number of large peptide molecules is effected under special conditions by proteolytic enzymes (79 to 84). It is interesting to note that Bailey (76) in the denaturation of edestin, and Holiday (85) in a study of the disaggregation of crystalbumin, both observed the liberation of tryptophane.

Different workers (23, 86) agree that the liberation of free thiol groups and denaturation are integral parts of the same process; an explanation of many puzzling facts is forthcoming if denaturation is regarded as a preliminary disaggregation followed by haphazard polymerisation of the split products. In the light of these conclusions it is not surprising to find that a number of workers have failed to effect reversal of the denaturation process (87 to 92).

Carbohydrates in proteins.—The carbohydrate-containing proteins fall roughly into two classes (93), those in which the carbohydrate residues are attached loosely by the formation of acid salts, etc., and are removed by comparatively mild treatment, and those in which the carbohydrate can be split off only by such drastic procedures as reaction with baryta or enzymic hydrolysis.

In the case of *B. dysenteriae* antigens Morgan & Partridge (94) have succeeded in dissociating the peptide and polysaccharide components, which are not antigenic separately, and in recombining them into an antigenic complex. The protein-carbohydrate linkage in this antigenic complex is comparatively easily ruptured but in a number of cases, e.g., serum and egg white proteins, the carbohydrate can be removed only by almost complete disintegration of the protein molecule; removal of the last traces of amino acids attached to the isolated carbohydrate is extremely difficult. The carbohydrate appears, therefore, to form an integral part of the protein molecule, and since it may constitute 10 per cent or more of the protein it is manifestly desirable that a clear formulation of its role in protein structure should be available. It is possible that the carbohydrate residue is of considerable physiological significance and may be of importance, for example, in the synthesis of serum proteins in the body.

In egg proteins Levene (95) has identified mannose and glucosamine, joined by a glycosidic linkage, but he could find no evidence of the presence of galactose. Stacey & Wooley (96), on the other hand, conclude that the carbohydrate residue in ovomucoid consists of a central core of three mannose units, to which are attached by glyco-

sidic linkages, seven N-acetyl glucosamine units and one galactose unit. They suggest similar structures for the carbohydrate residues in gastric mucin, pepsin, seroglycoid, etc., the carbohydrates being attached to the peptide portion of the molecule by ester linkages which engage mainly the 3-, 4-, or 6-hydroxy groups of the amino sugar.

Separation of the serum protein components of complement yielded a midpiece fraction containing 2.7 per cent, and an endpiece and fourth component containing 10.3 per cent of carbohydrate (97). Van den Ende (98) has described a serum gonadotropin preparation which was rich in carbohydrate and resembled seroglycoid.

The high carbohydrate contents of α- and β-globulin have been noted (99) and, in the serum proteins, a mannose : glucosamine ratio of approximately 2 : 1 is reported.

From human seminal plasma Ross, Moore & Miller (100) have separated a glycoprotein containing 26.8 per cent of reducing sugar (after hydrolysis), and 10.8 per cent of hexosamine, but no uronic acid.

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS

SECTION II

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General considerations.—The proteins fall readily into groups according to the function which they serve in the physiology of the organism, but frequently within a group, e.g., the haemoglobins, gross differences in solubility, crystal form, amino acid content, and immunological properties occur. Thus, proteins sharing a number of obvious properties reveal on closer inspection numerous differences which are attributable to species variation, and indeed there is evidence that they have a genetic character in the case of the haemoglobins. The picture is complicated by the facts that maternal and foetal haemoglobins are known to be different and that differences exist in the haemoglobin of the same individual living at different altitudes. This physiological adaptation may, of course, be a unique attribute of haemoglobin, but since this protein has received, for various reasons, more detailed investigation than any other, the possibility that foetal serum albumin, for instance, is a significantly different substance from adult serum albumin cannot be lightly dismissed. Sörensen's studies of a few purified proteins, which are still a model of careful investigation, further complicate the picture by leading him to the conclusion that these proteins in solution are never single chemical entities but systems of similar molecules in equilibrium with each other and with other constituents of the solution. His suggestion finds support in independent physical evidence that one protein in a mixture often affects the molecular size of another (1, 2).

The proof of protein purity is at once the disproof of the equilibrium theory, and discussion of the latter can only profitably centre around the criteria of purity of a protein, which have been the subject of a stimulating review by Pirie (3). Inability to melt or vapourise proteins precludes the application of several accepted methods of establishing purity, while crystallinity in them has no absolute significance although accepted as a useful means of purification. Constancy of chemical composition is important in the demonstration of purity, but

largely owing to the amount of work involved this has only been established for a few proteins and usually only with respect to a few atoms or residues which lend themselves to accurate estimation. The literature contains many records of variations in phosphorus, sulphur, carbohydrate, and nucleic acid content of proteins with successive crystallisations or precipitations.

Homogeneity of physical properties, especially molecular size and charge, is another important criterion, and much progress has been made towards a satisfactory proof of purity based on physical measurements. Unfortunately, these may generally only be applied to solutions of relatively restricted protein and salt concentrations though these adequately cover physiological conditions. Moreover, as in all osmotic methods, the relation between protein concentration and the property being measured is complex, and deductions of an absolute nature are limited to the state of the protein in a hypothetical solution of infinitely low concentration. From a practical point of view, however, it is in many respects more important to know whether serum albumin has the same molecular size in 2 per cent and 4 per cent solution than to know that different preparations of this protein have the same size at infinite dilution. The isoelectric point of a protein differs in different buffer solutions, and while, no doubt, the effects are due mainly to varying ion adsorptions they may in part reflect changes of a different nature, since a protein migrating as a single component at one pH may show a complex behaviour in a region little removed, the phenomenon being entirely reversible (4).

A third important and perhaps ultimate criterion of purity is based on solubility, for in conformity with the phase rule the solubility of a pure substance should be independent of the amount of solid phase. On this basis Kunitz & Northrop have deduced a high degree of purity for a group of enzyme proteins (5).

No protein has yet been shown to satisfy all the criteria of a stable chemical individual, viz., constant chemical composition, uniformity of molecular size and charge, and constant solubility. Perhaps β-lactoglobulin approaches most closely the ideal protein, since its solubility is almost independent of the amount of solid phase (6) and it is homogeneous in the ultracentrifuge and U-tube (7). The electrophoresis data were obtained by the rather insensitive light-absorption method, however, and a reinvestigation by a refraction method would be valuable in establishing its homogeneity. Other proteins satisfying one or more of these criteria and which may consequently be considered

as exhibiting a high degree of purity are chymotrypsinogen, which shows no solid phase effect (5), and bushy stunt virus, which sediments, diffuses, and migrates in an entirely homogeneous manner (8, 9).

It is important to know what proportion of the naturally occurring protein is isolated in "pure" form, since the fraction may be so small that the final apparent homogeneity may reflect more a rigorous chemical selection from a mixture than the occurrence in nature of a pure protein in significant quantities. The methods used in the isolation must also be critically examined to ensure that the product is not an artefact.

The protein chemist has now come to regard only a few laboratory manipulations as safe—dialysis, precipitation near neutrality, crystallisation, and separation in centrifugal and electrical fields. The dividing line between these and other procedures, such as contact with fat solvents, alcohol, drying, and acid precipitation, is arbitrarily drawn, and the question whether these methods bring about alterations in proteins has to be examined for individual systems.

Structure of conjugated proteins.—The exact nature of the linkages between haem and globin in haemoglobin, between carbohydrate and protein in egg albumin, or the phosphatide and protein of the serum proteins—to take a few examples—has so far resisted all attempts at solution. The prosthetic substance may be split off intact by acidification as in haemoglobin or by a combination of physical and chemical agencies as in the freezing of serum proteins with ether to remove lipoid (10), or the substance may only be liberated after enzymic proteolysis as in the removal of carbohydrate from egg albumin (11). Powerful forces are clearly involved in the association of proteins with prosthetic substances, yet in no case have these latter been isolated combined to a fragment of polypeptide by a recognisable chemical linkage.

In these circumstances it still remains uncertain whether the prosthetic substance and protein are linked by bonds of normal valence type or whether nonspecific surface or other attractions are involved. Physical studies indicate a readiness on the part of many protein molecules to disintegrate into a limited number of fragments, and in general, there is only a small number of molecules of prosthetic substance to each protein molecule. X-ray evidence suggests that the globular protein molecule is constructed of superposed laminae (12) and that prosthetic groups are evenly distributed in the molecule (13).

It is possible that the prosthetic substances may be interspersed between the laminae in relatively inaccessible positions and held there under the influence of non-specific surface forces from several directions.

The fact that the four haem groups in haemoglobin lie in one plane is shown (14) by measurements of refraction and light absorption along specific planes in the crystal of methaemoglobin. This elegant demonstration depends for its success on a favourable orientation of the molecules within the unit cell of the crystal. Whereas this is so in methaemoglobin crystals of the horse and sheep, Perutz (15) shows that it is not the case in crystals of horse oxyhaemoglobin grown in 20 per cent alcohol. In these the four molecules per unit cell are so arranged that the haemes, which are coplanar in the molecule, are not so in the crystal cell with the result that the crystal is optically negative. Thus, absence of birefringence or dichroism in a protein crystal does not preclude their presence in the molecule. The low density and corresponding looseness of structure make possible a reorientation of molecules within the cell without destroying the crystal form, and this is strikingly demonstrated in Perutz's oxyhaemoglobin by the reappearance of optical anisotropy on drying.

Granick (16) observed the diffusion of ferricyanide and hydrosulphite ions into guinea pig oxyhaemoglobin crystals and confirmed the relative looseness of the crystal structure. Channels at least 6 to 10Å in diameter are required to permit penetration of the ions, and these could contain some 20 per cent of the crystal weight as water. Since the advancing discolouration which he observes with ferricyanide arises from the interaction of this ion with haem groups, it appears that the latter must be arranged on the surface of the globins and facing the interstices between the crystal cells. The crystal shrinkage which accompanies drying of oxyhaemoglobin (15) is of the same order as occurs with insulin (17) and chymotrypsin (18), and, as with these, strong intramolecular x-ray reflections down to 7Å are observed in the dry crystals. This contrasts with the much greater shrinkage (up to 50 per cent) shown by methaemoglobin (14, 18) and β-lactoglobulin (19) and the absence of spacings below 13Å, in keeping with the view (20, 21) that drying, like denaturation, destroys the finer regularities in the molecule without involving any mass movements of atoms or amino acid residues.

So far a similar regularity in the arrangement of nucleic acid in the nucleoprotein molecule has not been demonstrated in spite of the

fact that the high density and specific ultraviolet absorption of nucleic acid suggest that its orientation in the molecule might be readily recognisable. Recent views (21) on the intramolecular structure of the tobacco mosaic virus postulate subunits of 11 x 11 x 11Å in cubic or hexagonal close-packing without reference to nucleic acid of which there is 5 to 6 per cent in the virus. The electron microscope shows, at least, that this nucleic acid is not concentrated locally in the molecule. Cohen & Stanley (22) report that various ribose nucleic acid preparations from the virus behave in a similar way to Astbury & Bell's desoxyribose preparations (23) and, in particular, that they tend to form long columns of flat nucleotides with a basal area approximately 100Å², i.e., the same as the subunits of Bernal and others. They favour the view that in each rod-shaped molecule of virus there are eight filaments of nucleic acid running from end to end of the rod on the surface since these are easily removed by heating. On the other hand, since inactivation of the virus by ribonuclease occurs without liberation of nucleic acid, Loring (24) suggests that the latter is not superficial.

Proteins concerned in blood clotting.—Almost all observers are agreed that clotting is a two-stage process, the first stage consisting in the activation of prothrombin present in the plasma to thrombin, and the second in the transformation of fibrinogen to fibrin under the influence of thrombin.

Cohn *et al.* (25) use 10 per cent ethanol in the cold to precipitate fibrinogen while Astrup & Darling (26), who find contraindications for the use of sodium chloride as a precipitant, prefer 0.28 saturated ammonium sulphate. Fibrinogen migrates in the electrophoresis tube as a single component between the β- and γ-globulins of human plasma (27), but its sedimentation rate in native plasma is still a matter for discussion (28). Purified fibrinogen sediments as an inhomogeneous material, and the asymmetrical molecules have a strong tendency to aggregate so that solutions are only stable for a few weeks at the most (26).

The contaminant of fibrinogen most difficult to remove is prothrombin, and although repeated salt precipitation is partly effective, the most satisfactory procedure is to adsorb it out of the plasma before the fibrinogen is precipitated. Astrup & Darling prefer colloidal tricalcium phosphate (29) to a number of adsorbents which have been used with success and obtain a fibrinogen which is prothrombin-free. With the exception of a precipitation curve, however, no evidence is

given for its purity in other respects. Mylon *et al.* (30) show that fibrinogen is selectively precipitated from plasma by protamin and compare this reaction as a method of estimation with the Cullen-Van Slyke recalcification of oxalate plasma.

Most methods of preparing prothrombin start from a crude fibrinogen precipitated with acid at pH 5.3. The prothrombin may be dissolved out of this precipitate with a dilute solution of calcium bicarbonate (31), or the precipitate may be redisssolved in saline and the prothrombin adsorbed on to colloidal magnesium hydroxide (32). Dialysis and further salt or acid precipitations (31, 33) give a solution which may change spontaneously to thrombin (31) but generally requires the addition of crude thrombokinase and calcium. Further purification of the thrombin depends on ammonium sulphate precipitations (31, 34) or on acetone precipitation and drying (35).

Prothrombin contains 14.03 per cent nitrogen, 3.8 to 4.7 per cent carbohydrate (33) and migrates with the albumin fraction although precipitating with the globulins between molarities 1.7 and 2.0 of phosphate buffer, pH 6.5 (36). Thrombin, on the other hand, contains 13.22 per cent nitrogen, 5.1 to 6.2 per cent carbohydrate (33), no phosphorus (34), and shows a solid phase solubility effect (31). No estimate of the molecular size of these proteins is available. Measurements of clotting times and clottable nitrogens show that increasingly active preparations are being obtained.

Proteins of egg white.—The complexity of the protein constitution of egg white is demonstrated in the survey by Longsworth *et al.* (37). Electrophoresis reveals the presence of seven components in the following proportions: egg albumin, 60.0; conalbumin, 13.8; ovomucoid, 14.0; globulins G_1, 2.8, G_2, 4.6, G_3, 4.3; and a mucin whose insoluble nature makes accurate estimation impossible. Only around pH 4.45 in the range pH 4 to pH 8 does dialysed egg white show peaks corresponding to all the constituents. These peaks have been correlated with fractions obtained by salting-out procedures.

Crystalline egg albumin migrates in a homogeneous manner at pH's in the regions of 3 and 11 while consisting at intermediate values of two components. Their ratio varies in different preparations but in any one sample is the same in the soluble fraction left after partial denaturation as in the original, thus disposing of the possibility that the variations rise from incipient denaturation.

Conalbumin prepared by acidification of the albumin fraction to pH 3.9 migrates as a single component at this pH but at pH 4.3 two

components are present in proportions which change slowly and reversibly with further increase in pH. In native egg white above pH 4 conalbumin migrates as a single substance with the mobility of one of these components. The disappearance of the other suggests an interaction with one or more of the egg white constituents. There is also evidence that egg albumin and ovomucoid may interact in egg white at pH 4.45 especially at high protein concentrations and low ionic strengths. From this complex system Longsworth & McInnes (38) proceed to study a simpler synthetic mixture, viz., egg albumin and yeast nucleic acid, with results which are probably of wider significance. At pH 5.34, where both substances have an appreciable negative charge, the pattern is essentially the sum of the patterns of the components, and the mobilities are normal. Below the isoelectric point of egg albumin (4.6) partial precipitation interferes with the study, but at 4.63, when egg albumin still has a slight negative mobility, the mixture behaves in an anomalous manner. In the anode compartment, a component migrating with the mobility of nucleic acid is found in lower concentration than in the mixture, while the second component is present in higher concentration than the egg albumin of the mixture and migrates with a mobility several times greater. The conditions in the cathode compartment are similar but in the reverse sense. The phenomena are explained by assuming the formation of an equilibrium complex between the components, and a comprehensive theoretical treatment of the effect of dissociable complex formation on the electrophoretic behaviour of mixtures is developed.

The mobilities of egg albumin at 0° over the range of pH 3 to 11.8 in buffers containing only monovalent ions is considered (39) in relation to the titration curve corrected to the same temperature. Over this range the proportionality factor relating charge to mobility deviates by not more than 7 per cent from a mean value. Such a constancy implies that the frictional coefficient is equally constant and in turn that the diffusion constant does not change. New determinations of the diffusion constant over a wide pH range show this to be the case.

Composition of normal serum.—Normal human serum and plasma show a remarkable constancy of protein composition as is evidenced by the agreement in the data from several laboratories for the quantities of albumin, α-, β-, and γ-globulins, and fibrinogen present (Table I). In a study of the effects of various buffer salts, using a U-tube of twice the normal length, Longsworth (40) shows the presence of two α-globulins in normal human serum (α_1 and α_2). In all previous

analyses the first of these has been included in the albumin figure. The amounts of the different constituents for a single plasma sample, as percentages of the total protein, are: albumin 43.8, α_1-globulin 6.4, α_2-globulin 10.3, β-globulin 22.1, γ-globulin 9.9, and fibrinogen 7.5. A comparison of these figures with the data of Table I shows certain inconsistencies: the sum of the albumin and α_1-globulin values is lower

TABLE I

PROTEIN CONSTITUENTS OF NORMAL HUMAN SERUM AND PLASMA*

Number of Samples Investigated	Albumin	Globulins			Fibrinogen	Reference
		α	β	γ		
1	64.2	8.5	16.8	10.5	...	(41)
4	64.1	7.7	14.7	13.5	...	(48)
6	62.5	5.0	11.5	21.0	...	(49)
6	66.3	4.5	12.2	17.0	...	(47)
2	59.9	8.4	12.6	13.8	5.4	(48)
12	61.8	7.8	13.6	11.7	5.0	(44)
—	62.5	7.0	13.2	11.6	5.7	(42)

* Expressed as percentages of the total protein.

than that found for albumin alone and the amount of β-globulin is greater than that previously reported. Longsworth shows that this is not due to specific buffer effects.

β-globulin is shown by Svensson (41) to consist of two components, and Luetscher (42) finds these to account for 4.8 and 8.4 per cent, respectively, of the total serum protein. He also finds (43) that normal serum albumin which migrates as a single substance at pH 7.4 consists of two components in the ratio of approximately 2:1 at pH 4.0. Moore & Lynn (44) examine statistically their own and other data and calculate limits for variations in the composition of normal human serum and plasma.

The serum of normal horses shows a much greater variability in protein constitution than human (45, 46) and also differs from it in exhibiting a lower proportion of total serum protein as albumin. Svensson (46) gives data also for normal cow, swine, and rabbit serum and Seibert & Nelson (47) for normal rabbit serum.

Chemical status of serum proteins.—Serum albumin from new species and by new methods of preparation (25, 50, 51) has not affected significantly the characteristics of this group. The serum albumins remain as an assemblage of proteins with molecular weights in the region of 70,000 and isoelectric points close to pH 4.8. Members of

the group which have hitherto resisted attempts at crystallisation appear to be yielding to more enlightened handling, notably to more effective preliminary removal of α- and β-globulins and lipoid by salt or ethanol fractionation (52). The carbohydrate content of different preparations, some from the same serum, is variously 0 (53, 54), 2 (53), 5.5 (55), and 10 per cent (56). The independent status of these proteins in native serum must still be regarded as *sub judice*. While there is no doubt about the homogeneity in respect of molecular size of serum albumins over a wide pH range, electrophoresis shows a single boundary only outside the pH range 4 to 6 (5, 43). Within this range the pattern is complex and often different in the two limbs of the U-tube for reasons still to be explained.

While the carbohydrate content of serum albumin has been receiving so much attention, little or none has been paid to the lipoid which, in the case of human albumin separated by electrophoresis, represents approximately two molecules of cholesterol and two of phospholipids per albumin molecule (58). The evidence that this lipoid is an integral part of the molecule and associated with it in stoichiometric proportions is, however, not any more convincing than it is in the case of the carbohydrate.

Similar studies have been made on the globulin fractions isolated by electrophoresis, of which the γ-globulin is better defined than the α- and -β. The γ-globulin shows an abnormal degree of boundary spreading in the ultracentrifuge and the U-tube, and the solubility is far from constant (52, 57) It is not known whether this behaviour arises from an essential variation in molecular properties or a random association of low molecular serum constituents with protein molecules of uniform size. It is of interest in this connection that the molecule is highly asymmetrical (59) and would therefore be expected to interact strongly with other ions present. Blix *et al.* (58) find the human γ-globulin molecule to be associated with 1 to 2 molecules cholesterol, 2 molecules phospholipid, and 12 to 14 molecules dimannose.

The isolation of α- and β-globulins is a more difficult matter whether by electrophoresis (58) or by salt or ethanol fractionation, (25, 50) and there is little evidence for the ultimate purity of the materials so obtained. A high proportion of carbohydrate and lipoid is associated with both fractions; thus α-globulin contains 6 per cent carbohydrate (as mannose), 4.5 per cent cholesterol, and 7.2 per cent phospholipid, while β-globulin contains the same amount of carbohydrate but 8.6 per cent cholesterol and 10 per cent phospholipid (58).

Thus altogether some 18 per cent of the α-globulin and 25 per cent of the β-globulin fraction is non-protein in nature. The composition of the remainder is not known; it may represent specific α- or β-proteins or may consist wholly or partly of albumin or γ-globulin.

It is reported (58) and confirmed (10) that coarsely dispersed lipoid particles in human serum (chylomicrons) migrate usually with the β-globulin boundary and confer a characteristic turbidity on this fraction. These particles are prevented from aggregating by association with a serum constituent not soluble in ether and the protective value of which is destroyed by freezing to below the eutectic temperature of serum. This substance, which is presumably β-globulin, appears also to stabilise a variety of colloidal materials—cholesterol, mastic, Sudan III—when these are added to lipoid-free serum (60). In the presence of lipoid, however, there seems to be a deficiency of the protective substance, and foreign particles are stabilised by α-globulin or by a composite surface layer in which α-globulin or even albumin may participate with β-globulin. Other relevant facts which cannot be fully assessed at the moment are that extraction of serum with acetone in the cold causes the α-boundary to disappear (60), while exhaustive ether extraction with freezing (10) removes only some 65 per cent of the lipoid (mainly cholesterol and its esters), leaving significant amounts of ether-soluble material which must be protected against the action of ether in a unique manner. The extracted lipoid appears to be associated mainly with the β-globulin fraction.

Electrophoresis of pathological sera.—It would appear opportune to summarise the information obtained in the last few years on this subject, which has not so far been reviewed *in extenso*. The serum from patients showing a variety of pathological conditions, with fever in common, all show an enhanced α-globulin content (48). The increased α-globulin in pneumonia, first demonstrated by Blix (61), is confirmed by Luetscher (42). In acute rheumatic fever the γ-globulin is also increased (48, 62).

In plasma from two cases of multiple myelomatosis Longsworth *et al.* (48) found a marked increase in β-globulin, while the plasma from a third was quite normal. Four of Kekwick's sera from cases of this disease (49) showed substantially increased γ-globulin by electrophoresis and only one globulin boundary in the ultracentrifuge, while in a fifth, showing altogether five boundaries in the ultracentrifuge, an excess of the β_2-globulin was demonstrated in the electrophoresis apparatus. Gutman *et al.* (63) have attempted to correlate the serum

electrophoresis findings in this and other hyperproteinaemic conditions with protein concentrations determined by Howe's salting-out technique. In most of these both euglobulin and pseudoglobulin I are increased. In some of the myeloma cases, however, the increase only involves the pseudoglobulin I or the pseudoglobulin II or even the albumin. Electrophoresis showed increased β- or γ-, but occasionally also a protein designated M, migrating between the two. Some quite normal sera have been found, thus justifying a classification of the disease into normal and abnormal serum types. In the latter the serum changes consist alternatively in an increase in γ- corresponding to an increased euglobulin, or to a variety of electrophoretic and salting-out changes. The appearance of the various electrophoretic patterns encountered can be simulated by the addition of Bence-Jones urinary proteins, differing in properties from case to case, to normal human serum; Gutman *et al.* suggest that some of the anomalous serum patterns observed are due to the presence of these proteins in the serum.

Nephrotic sera show characteristic increases in α- and β-globulins (42, 64) and a low proportion of albumin. At pH 4 normal human albumin consists of two components, and Luetscher demonstrates that the albumin separated from nephrotic sera at this pH contains similar components but in different proportion. The lipoid in nephrotic sera is less stable than in normal serum since it can be extracted with ether at room temperatures; the β-globulin in the extracted serum encloses a smaller area than in the unextracted. Urinary protein from cases of nephrosis is similar in nature to normal human serum, but the albumin may be increased to as much as 90 per cent of the total protein.

The serum protein changes in tuberculosis are investigated by Seibert & Nelson (47) who find in rabbits that there is no marked increase in total serum protein but electrophoresis shows a decreasing albumin and increasing α-globulin as the disease progresses, with the appearance of a new "X" component of slightly higher mobility than albumin. The mobility of the new component corresponds to that of tuberculin protein when added to normal rabbit serum. In human tuberculosis both α- and γ-globulin are increased and an "X" component is present. There is evidence that the increase in γ-globulin is associated with resistance to the disease.

The erythrocyte sedimentation rate and electrophoretic constitution of corresponding serum or plasma have been examined by Shedlovsky & Scudder (65) in normal subjects and in various pathological conditions. There is a reasonable correlation between the sedimenta-

tion rate and α-globulin concentration, both increasing when there is considerable tissue inflammation or destruction from any cause.

The behaviour of pleural exudates and ascitic fluids in a variety of pathological conditions (pneumonia, tuberculosis, cirrhosis) has been compared with that of the corresponding plasmas (62). The effusion generally contains the same components as the plasma, and while the globulin proportions remain relatively constant, the proportion of albumin may be increased. Where the serum has an abnormal constitution this is reflected in the behaviour of the effusion.

Antibodies, toxins, and antitoxins.—The original observations of Tiselius & Kabat (66), that the antibody to egg albumin in the rabbit migrates with the γ-globulin and the antipneumococcal antibody in the horse with a component between the β- and γ-globulins, have stimulated studies of antisera of many kinds with the U-tube and the ultracentrifuge. The pneumococcus antibody in the cow, horse, and pig has a molecular weight of 990,000 (67), normal horse serum containing a small quantity of a globulin of this weight, while in humans, the rabbit, and monkey the molecular weight is that of normal serum globulin. In the U-tube the behaviour is not quite consistent even within a species. Thus, of a number of sera from horses immunised against a pneumococcus all showed an increased γ-globulin except one which had a component migrating between the β- and γ-globulins (68).

More consistent results are obtained in diphtheria immunisation in which the serum antibody always has the weight of normal serum globulin and migrates partly with the β_2- and partly with the γ-globulin (45). These two globulins are known to possess different characteristic flocculation times and *in vivo/in vitro* activity ratios. The B_2-globulin, with which is associated 90 per cent of the antitoxic activity of the serum, is separated by Pappenheimer *et al.* (69) by a combination of salt-precipitation and electrophoresis; it is homogeneous in sedimentation, diffusion, and migration and 43.5 per cent precipitable with toxin. The weight of the molecule is 184,000, but on digestion with pepsin it splits into active and inactive fragments. The active material, which is separated from the inactive by heat precipitation in a suitable salt environment, contains a homogeneous protein of molecular weight 98,000; considerations of symmetry indicate that it has been formed by binary fission of the parent molecule in a plane normal to the long axis. All the antitoxic activity is concentrated in this fraction, but since the specific precipitability is unchanged, the proportion of active to inactive globulin molecules must be the same as in the

original antiserum. The flocculation time of the new product is, however, much less for obscure reasons, and a greater part of the carbohydrate of the parent globulin passes to the active split product (70).

Highly active diphtheria toxin (0.00046 mg. N/Lf. unit), obtained by salt fractionation of culture filtrates, behaves as a homogeneous material in the ultracentrifuge and U-tube (70, 71). The sedimentation rate is constant over the pH range 5.6 to 10.1 which corresponds with the stability range determined by toxicity assay. Below pH 5.6 the toxin is precipitated, and at 10.8 the sedimentation constant begins to fall. The molecular weight, determined from sedimentation and diffusion data, is 74,000 (70) and the isoelectric point from observations of the behaviour of quartz-coated particles is at pH 4.1. Since the toxin becomes insoluble below pH 6 (71) this is probably the isoelectric point of the denatured toxin.

In studies of the toxin-antitoxin reaction Pappenheimer *et al.* (69) used purified toxin and β_2-antibody globulin of molecular weight 184,000. At the equivalence point the floccules contained one molecule of toxin to every two of antitoxin, while in the region of antibody excess the ratio was one of toxin to eight of antitoxin and in the region of toxin excess, two of toxin to one of antitoxin. These represent maximum valence figures for toxin and antitoxin in relation to each other, and in general the reaction appears to rest on a stoichiometrical basis.

It is also now established that the reaction is fundamentally a neutralisation without destruction of specific activities, and since both components are precipitated in the process, it offers a unique means of separating them from other constituents of the serum and culture medium. Petermann & Pappenheimer (70) digest the washed floccules from a mixture of equivalent amounts of toxin and antitoxin with pepsin and fractionate the digest with ammonium sulphate to obtain a homogeneous globulin which is 77 per cent precipitable and has a molecular weight of 113,000. Northrop (72) chooses to digest the acid-treated floccules with trypsin at pH 7.2 and to isolate the antibody by precipitation between 0.33 and 0.65 saturation with ammonium sulphate. A portion of this amorphous but highly active material precipitable between 0.50 and 0.65 saturation exhibits constant solubility, is 90 per cent specifically precipitable, and has an antitoxic activity of 700 to 900 Lf. units per mg. protein. From this fraction the antitoxin is crystallised as thin plates in ammonium sulphate at pH 6.8, the yield of thrice-crystallised product representing only 0.3 per cent of the activity of the original antiserum. Crystal-

lisation appears to cause some decomposition resulting in a decreased solubility. The crystalline protein sediments and migrates as a homogeneous material of molecular weight 90,500 and isoelectric point at pH 7.0. The degree of boundary spreading in $M/15$ veronal is abnormal but is reduced after addition of $M/20$ calcium chloride. This leads Northrop to the conclusion that the spreading does not reflect an essential variation in particle charge but is an endosmotic effect (72, 73). The trypsin-split product shows no increase in carbohydrate content in contrast to the pepsin product.

From these and other studies it is at least clear that the technique of enzymic digestion of specific precipitates, followed by heating and salt fractionation, offers great possibilities for the preparation of efficient protein antibodies. The effect of peptic digestion alone on normal and immune sera is studied by van der Scheer *et al.* (74), who find in diphtheria and tetanus antitoxic sera that the albumin is broken up and the β_2- (their "T") component merged with γ-globulin, while in antipneumococcal serum the prominent γ-globulin component disappears. With regard to the effect of heat alone it is obvious from the increased Tyndall dispersion of heated serum that aggregation plays an important part in the changes which occur. Electrophoresis of normal horse serum after heating to 65° for 15 minutes (75) shows evidence of complex formation between the albumin and γ-globulin. The complex is heavy enough to be deposited in centrifugal fields, which do not affect the serum globulins, and migrates close to the β-globulin. It is also precipitated from salt-free solutions at pH 6. Heating albumin alone causes no significant change, but heating salt-fractionated globulin results in a marked aggregation and change of mobility to a value which is, however, less than that of the complex formed in serum by heating. Kleczkowski describes the formation of protein complexes on heating rabbit serum albumin with various proteins (76). Thus, when the albumin and euglobulin of the rabbit are heated separately and mixed under controlled conditions, salt precipitations demonstrate the formation of complexes. The presence of albumin-antibody complexes in heated antisera is also demonstrated (77) by precipitin reactions using plant virus antigens.

Krejci *et al.* (78) find five components by electrophoresis of crude culture filtrates of a strain of streptococcus associated with scarlet fever, but only one of these has toxic and erythrogenic properties. The active material sediments and migrates as a homogeneous substance of molecular weight 27,000. It contains 15.2 per cent nitrogen,

is heat-coagulable, easily inactivated by surface denaturation, and is highly toxic.

A variety of substances are also present in culture filtrates of human tubercle bacillus, one of which—tuberculin protein—determines the activity in skin tests. Below pH 5.0 electrophoresis shows that this protein migrates as a complex with nucleic acid present in the filtrate, whereas above this pH the two migrate independently (79). It is accordingly more likely that separation can be effected with ammonium sulphate at pH 7 than by the earlier procedure of precipitating the protein with trichloroacetic acid. Filtrates from cultures on synthetic media, concentrated and pressure-dialysed on the ultrafilter against buffer at pH 7.3 and half-saturated with ammonium sulphate, yield a protein containing 1.7 per cent nucleic acid and 2.9 per cent carbohydrate (80). This protein migrates and sediments in a uniform manner and has a molecular weight from sedimentation and diffusion data of 14,500. Using a different synthetic medium, a less homogeneous product of smaller molecular weight (10,500) is obtained. Both molecular weights are smaller than earlier estimates (32,000) (81), and further investigation is desirable.

Amino acid composition and acid-base equilibria.—Chibnall (82) publishes new analytical figures for the amino acid composition of edestin, β-lactoglobulin, egg albumin, and insulin with the claim that the values for the more important constituents—aspartic and glutamic acids, arginine, histidine, and lysine—are correct to 1 to 2 per cent. As shown in Table II there is an excess of free amino groups over the number corresponding to the lysine content in five proteins. In

TABLE II

NUMBER OF FREE AMINO AND CARBOXYL GROUPS IN SOME PROTEINS

Protein	Mol. Wt.	Free Amino Groups		Free Carboxyl Groups	
		By Titration*	By Analysis†	By Titration*	By Analysis†
β-lactoglobulin ...	42,000	35–37	28	62–63	61
Egg albumin	43,000	21	15	49	45
Insulin	35,000	21	3	..	..
Edestin	50,000	12‡	8	..	..
CO-haemoglobin ..	67,000	53	37	..	..

* Mainly from Cannan *et al.* (83, 84), quoted by Chibnall (82).

† From Chibnall (82) based on lysine and dicarboxylic acid contents.

‡ Three of these are ascribed to edestan formation under the conditions of the Van Slyke amino-nitrogen estimation.

accordance with a suggestion made by Cannan (85), each group in excess is presumed by Chibnall to be a terminal α-amino group so that the molecule of β-lactoglobulin which has nine in excess is regarded as consisting of nine polypeptide chains, egg albumin of four, insulin of eighteen, edestin of one (per sub-molecule of wt. 50,000), and haemoglobin of sixteen. Since each chain must also have a free terminal α-carboxyl group, egg albumin should show four titratable carboxyls in excess of the number indicated by the dicarboxylic acid content, as in fact it does. However, in the only other case in which carboxyl data are available, viz., β-lactoglobulin, there is an excess of only one carboxyl where nine are required. It is suggested in this case that nine terminal carboxyls are present but that compensatory eight side-chain carboxyls are masked by ester cross-linkages involving the ω-carboxyls of dicarboxylic acids; three types of possible linkage are described. It is implied that hydrolysis of β-lactoglobulin will result in the liberation of eight more carboxyl than amino groups, but in view of the hundreds of peptide linkages which are simultaneously broken, ordinary analytical methods are not likely to detect this excess. In support of the view that the protein molecule contains multiple polypeptide chains, Chibnall cites x-ray evidence by Astbury (12) that egg albumin comprises four superposed laminae and by Crowfoot (20) and Bernal (86) that insulin has eighteen submolecular units.

Cannan (87), however, is of the opinion that neither in egg albumin nor β-lactoglobulin are there more than one or two free α-amino groups present in the molecule, a conclusion based on a refined analysis of titration curve data. Chibnall's conclusions as to the number of polypeptide chains in these proteins must therefore be treated with reserve, and the problem of the nature of the excess free amino groups, indicated by titration and Van Slyke estimations over those expected from amino acid analysis, regarded as still an open one.

Cannan also discusses extensively the more important problems arising in the determination and interpretation of the acid-base titration curves of soluble proteins. The devices adopted in correlating segments of the curves with the ionisation of definite groups in the protein and their quantitative estimation from stoichiometric points on the curves were discussed in some detail in last year's review (88). A theory is developed by Cannan which describes quantitatively the dissociation curves of proteins in terms of a single intrinsic dissociation constant, K_o, applicable to all similar groups and including expres-

sions allowing for the effects of electrostatic interaction and ionic strength of the medium in producing a spread in the apparent dissociation constant, K′, for a polyvalent ampholyte. For egg albumin the values of pK_o which were found to apply were 4.5 (carboxyl), 6.7 (imidazole), and 10.0 (amino). For β-lactoglobulin the values 4.5 (carboxyl) and 6.7 (imidazole) were found, but the theory in this case fails to account for the slopes and positions of the amino segments of the curve. The reason for the discrepancy between the values of pK_o for the carboxyl groups in the two proteins is not clear.

It is deduced that increase in size of the molecule will widen the pH span over which groups of a kind dissociate. The difference in shape between a dissociation curve in the presence of neutral salt at constant ionic strength and the curve for a protein in the absence of salt is attributable to the varying contributions to the ionic strength of the protein ions themselves in the latter case, this contribution not being significant in the first instance.

Mobility and charge.—It is established that most proteins when isoionic, i.e., combining neither with H^+ nor OH^-, still possess a free charge by virtue of which they migrate in the electrophoresis apparatus. This charge is attributed to the adsorption of ions other than these two (89), and for purposes of interpreting titration data in terms of the electric properties of the molecule and the medium, the device is now generally adopted of moving the titration curve bodily in the direction of the valence axis until the isoionic and isoelectric points coincide. In the case of β-lactoglobulin (84) these two points are the same so that deductions based on the behaviour of this protein are free from assumptions regarding the validity of this axial adjustment. It is also established that both titration and mobility curves depend on the concentration of buffer salts, and any change in ionic strength is responsible for a rotation of these curves about this arbitrary point of coincidence. The contribution of the protein itself to the ionic strength cannot be dismissed, especially at low concentrations of buffer salts.

As a special case the behaviour of dilute protein solutions containing concentrations of buffer salts may be considered and the results extrapolated to zero ionic strength. For egg albumin, serum albumin, and β-lactoglobulin Gorin & Moyer (90) find that the net charge deduced from equivalents of acid or base bound agrees well with that calculated by means of Stokes law and a simple expression for the velocity of a charged particle in a dielectric medium under the influence of an external field. In the special circumstance of zero ionic strength considered here the protein molecule is visualised as a free

ion and the measure of agreement obtained indicates that this must be so.

In the presence of appreciable concentrations of salt ions, however, the protein molecules attract an atmosphere of oppositely charged ions to form a diffuse double layer, the effect of which is to screen them from the applied field and so to reduce their mobility. By application of the Debye-Hückel theory an expression is derived (91) for the mobility (u) in terms of the net charge (Q), viz.,

$$u = \frac{Q}{6\pi\eta r} \cdot \frac{f(K\alpha)}{(1 + K\alpha)}$$

where η is the viscosity, r the radial distance to the surface of shear, α the distance of closest approach of the ions to the particle, and K is the square root function of ionic strength. In the hands of numerous investigators (39, 83, 90, 92) this equation provides values for mobilities from measured equivalents of acid (base) bound which are greater than the measured values and, in buffers of ionic strength 0.1 and over, may be two or three times greater (90).

On the other hand, there is evidence that the equation gives true values for mobilities when the net charge is computed from membrane potentials instead of equivalents of acid bound. Unfortunately, information of membrane potentials is scarce but Adair & Adair (93) have shown that this agreement exists in the case of carboxyhaemoglobin over the pH range 6 to 10 and at ionic strengths up to 0.1; Tiselius & Svensson (94) have demonstrated the same for egg albumin at a constant pH of 7.1 and various ionic strengths up to 0.2. It is desirable that these investigations should be continued and extended to other proteins, especially in view of Longsworth's cogent remarks (39), viz., that they substantiate a theory which presumes that the egg albumin molecule is spherical, which it probably is not (95), and that the protein does not affect the activity coefficients of the buffer ions, which it probably does (96).

Gorin (97) has modified the equation to take account of the finite size of the buffer ions and the shape of the protein molecule, but these modifications, although important in themselves, are not sufficient to account for the discrepancy between mobilities and acid or base bound as Gorin & Moyer (90) point out. These authors favour Steinhardt's view (98) that undissociated acid or base is bound to the protein, giving rise to higher estimates of valence than are actually the case while not affecting membrane potentials.

Reversible boundary spreading in electrophoresis.—Gradients in pH and electric field are sometimes present at the migrating boundary,

but their effects can be reduced to negligible proportions by working at low protein concentrations and high ionic strength. In these circumstances the ascending and descending boundaries have the same shape. A considerable amount of reversible boundary spreading still occurs under these conditions. The quantitative aspects of this have been considered by Sharp *et al.* (99), who find the following relation to hold: $\frac{\Delta\sigma}{E\Delta t} = H$, where $\Delta\sigma$ is the change in standard deviation of the curves occurring in time Δt at the field strength E and H is a constant determinable experimentally. It is deduced on the assumption of a distribution of mobilities about a mean value U that such a relation is to be expected, and H is referred to as the heterogeneity constant. Low values of H are consistent with a narrow distribution of mobility, that is, a high degree of electrochemical homogeneity.

Miscellaneous.—A nomogram for rapidly computing sedimentation data has been devised by Schachman (100). Lauffer (101) describes an arrangement for accelerating dialysis of protein solutions.

The molecular weight of insulin is redetermined by diffusion and sedimentation, the revised value being 46,000 (102); it migrates as a single component though the mobility varies according to the method of solution (103). The gonadotrophic hormone of pregnancy urine has a molecular weight of 100,000 (104), and crystalline crotoxin is homogeneous in the U-tube and isoelectric at pH 4.71 (105). The molecular weight of crystalline ribonuclease is 15,000 (106), while a protein antibiotin factor from egg white having a sedimentation constant of 4.7 is homogeneous in the U-tube with an isoelectric point near pH 10.0 (107).

Dills & Nelson (108) have prepared a protein from milk containing organically bound copper, and Wawra & Webb (109) have isolated a new prosthetic substance, the chalcone of hesperidin, from combination with proteins. Schales (110) reports that a new preparation of renin from pig kidney has the solubility properties of a pseudoglobulin. The magnetic behaviour of catalase is examined by Michaelis & Granick (111).

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR

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Since publication of the last review on this subject, present conditions have caused both curtailment of activity in the sulfur field and inaccessibility of many foreign journals. Nevertheless, restrictions in space still compel omission of a number of borderline topics.

Reactions of sulfur compounds.—A new and convenient method of synthesis of *dl*-methionine has been described (1) using as the starting point, α-acetobutyrolactone. A method has also been published for the synthesis of methionine containing radioactive sulfur (2). The formation of S-cysteine sulfonates and of thiol groups by the action of sodium bisulfite on the disulfide linkages of wool has been reported (3), and similar studies have been made of the reaction of formaldehyde on the cystine disulfide linkages in wool (4, 5). The formation and reactivity of 2-methyl thiazoline have been studied (6). This compound is highly unstable and is easily denatured by heat, producing thiol groups and a peptide linkage.

The well-known reaction between cyanides in alkaline solution and cystine has been shown to proceed also at pH 5. Although much slower it follows the same course, producing one mole of cysteine per mole of cystine, and is obviously not a reduction but a hydrocyanolysis. The oxidized form of glutathione reacts in an analogous way (7).

Investigations have been reported of some *in vitro* reactions of radioactive sulfur (2). Free radioactive sulfur with cysteine is reduced to hydrogen sulfide with formation of cystine. All radioactivity remained with the hydrogen sulfide and none appeared in the cystine. No evidence of reversal of this reaction was found even in alkaline solution. Thus the validity of this method of labeling sulfur in such reactions appears to be well supported.

Cystine can be reduced by means of catalytic hydrogenation using a synthetic palladium high polymer catalyst (8). This catalyst shows much less tendency to be poisoned by the sulfur than does ordinary palladium black.

Analytical methods.—Various refinements of existing analytical methods have been described and some new methods suggested. The use of the polarograph in the determination of cystine continues to invite investigation. Kolthoff & Barnum (9) have made a careful study of the reduction of the disulfide linkage of cystine at pH 1. The irreversibility of the reduction of cystine, first reported some years ago, has been confirmed by these authors who describe the details of the determination. Figures for the cystine content of normal urines (40 to 80 mg. per twenty-four hours) obtained by polarographic determination correspond satisfactorily with those obtained from colorimetric methods (10).

The employment of cuprous oxide for simultaneous reduction of cystine and precipitation of the cuprous mercaptide with subsequent use of the Sullivan colorimetric reaction has been described (11), and this same reagent (cuprous oxide) has also been employed (12) to effect quantitative removal of cystine from keratin hydrolysates without leaving behind an excess of inorganic salts, as in the usual methods for cystine preparation. However, when used as a precipitant of sulfhydryl compounds with later estimation of the latter by the nitrogen content, the precipitation of other forms of nitrogen (purines, etc.) must be taken into account. Determination of the sulfur content of such a precipitate is much safer (13). Following the report by Ross (14) that the use of the Baernstein method for cysteine, as modified by Kassell & Brand (15) on the protein of tobacco mosaic virus, gave results according better with the total sulfur content than did the results of the Sullivan method. Hess, Sullivan & Palmes (16) investigated the application of the Sullivan method to this protein. They concluded that satisfactory results are obtained if humin formation is prevented preferably by the use of hydriodic acid. A modification of the Sullivan method for the purpose of estimating both cystine and cysteine in mixtures has been proposed (17). It is based on the difference in molar ratio of cystine used to cysteine formed when the reagent used is sodium amalgam instead of sodium cyanide.

A new colorimetric method for cystine and cysteine is stated by the author to be capable of determining from 0.01 to 0.20 mg. cystine (or cysteine) per ml. with an average accuracy of ± 3 per cent (18). An oxidation method for cysteine employing *o*-iodosobenzoic acid has also been described (19).

Procedures for the more accurate determination of methionine are being steadily improved. Beach & Teague (20) describe the determi-

nation of methionine sulfur as barium sulfate. The methionine, demethylated by hydriodic acid, gives the thiolactone. The latter is not precipitated by cuprous oxide unless the ring is previously opened by alkali, whereas the cysteine resulting from the cystine of the protein precipitates as the cuprous compound without such treatment. Values on several proteins, both purified and crude, agree well with the results obtained by other procedures.

An investigation of the reaction between methionine and sodium nitroprusside under alkaline conditions, followed by acidification, has resulted in the proposal of a new colorimetric method (21) by means of which the authors report good methionine values for such proteins as casein and edestin.

A third departure in methionine determination has been proposed by which the amino acid is oxidized by a known excess of hydrogen peroxide in perchloric acid solution, the excess being determined by iodometric titration (22). Under these conditions a small amount of interference is encountered with tryptophane, cystine, and cysteine and practically none with other amino acids. The subsequent liberation of iodine from the iodo sulfonium iodide formed from methionine and iodine has been suggested for the estimation of methionine by Lavine (23). In connection with a study of the oxidation of sulfur in organic and biological materials, Lefevre & Desgrez (24, 25) have suggested certain modifications of the Baernstein method for methionine.

In a discussion of various methods for the quantitative oxidation of cystine and other forms of sulfur, Callan & Toennies (26) describe a procedure, using alkaline permanganate for the preliminary oxidation of the compound, in which the sulfur of cystine and certain cystine derivatives is recovered with satisfactory accuracy but no sulfate is formed from the methionine. Hoagland (27) has reported a micromethod for sulfates and for sulfur after oxidation to sulfate, in which precipitation as benzidine sulfate is used followed by combustion of the carbon and manometric measurement of the resulting carbon dioxide. A colorimetric determination of benzidine sulfate by its reaction with dimethyl-*p*-aminobenzaldehyde has been described (28, 29), but the method labors under the disadvantage of producing a rather unstable color. Guillaumin (30, 31) calls attention to various errors inherent in barium sulfate precipitation from deproteinized serum and concludes that different techniques give such variable results that it is uncertain whether sulfuric esters occur at all in serum.

However, a very involved procedure for the microdetermination of ethereal sulfates in blood and pus, published by Norberg (32), is claimed to determine down to about 0.05 mg. sulfur in this form with an accuracy of ±10 per cent. A microsedimentation method has been applied to barium sulfate from serum (33). Thallium has been substituted for lead in the precipitation of the "labile sulfur" of proteins and its action was found to be practically identical (34). Hydrogen sulfide in stomach contents of experimental animals has been determined, its evolution being caused by nascent hydrogen (35). The Evelyn photoelectric colorimeter has been employed in the determination of thiocyanate as the ferric salt (36) in plasma, serum, and urine.

A method for the determination of lanthionine is based on its reaction with hydriodic acid, producing one mole of cysteine to which the Sullivan colorimetric method is then applied (37).

Newly isolated or synthesized sulfur compounds.—A noteworthy advance in our knowledge of the effect of alkalies on the cystine group of proteins has been afforded by the isolation of a thio ether, β-amino-β-carboxyethyl sulfide, from the acid hydrolysate of wool that had been previously boiled for one hour with 2 per cent sodium carbonate solution (38). The effect of alkalies on cystine and cystine-containing proteins has attracted attention for many years, and the isolation of this thio ether promises to open a valuable field of investigation. The authors suggest the name "lanthionine" to indicate its origin from wool and its sulfur content. Prepared as above it is optically inactive.

A prompt report of its synthesis by du Vigneaud & Brown (39) followed. It was obtained by treating *l*-cysteine with methyl *dl*-α-amino-β-chloropropionate hydrochloride, a procedure analogous to that used for the synthesis of S-(β-amino-β-carboxyethyl)-homocysteine, the mixed thio ether of α-alanine and α-aminobutyric acid (40). The product corresponded entirely with that isolated from alkali-treated wool.

Lanthionine was also isolated from other keratins and it was shown that 0.1 *N* sodium hydroxide or dilute sodium sulfide could be substituted for the sodium carbonate (41). Horn & Jones (42) have also reported the isolation of a crystalline compound from vetch grown on seleniferous soil which appeared to consist of one mole of lanthionine to two of the corresponding selenium compound. It is interesting to note that investigation of such a mixed sulfur and selenium compound (43) first directed the attention of these investigators to the thio ether.

d-, *l-*, and *dl*-Isomers were also prepared by Brown & du Vigneaud (44) and their properties recorded. In contrast to the high specific rotation of cystine, the thio ether shows a specific rotation of only ±8. These, together with the meso form already reported (38), complete the series of possible optical isomers of this compound. Mesolanthionine has, by methods similar to the above, been isolated from insulin (45) and other proteins (46). From human hair the *dl*-form, as well as the meso, has been obtained (47).

The metabolic behavior of this substance may be of considerable moment. It has been suggested (48) that exposure of any cystine-containing protein to even the mildly alkaline conditions of the intestinal tract might produce enough lanthionine to make its nutritional status a matter of more than academic interest. Moreover, reinvestigation of the alkaline decomposition of cystine in the light of the recent work on lanthionine should be worthy of attention. The mode of action of alkalies (49) and of reducing agents (50) on the cystine linkage in keratin fibers has led Stoves to postulate some reactions which could provide a possible explanation for the formation of lanthionine. Further studies on the alkaline decomposition of cystine have reported the identification of uvitic, uvitonic, and thiolactic acids as decomposition products (51).

The existence of addition compounds of dehydroascorbic acid with certain sulfhydryl compounds (cysteine, thioglycollic acid, and reduced glutathine) in equimolar proportions has been demonstrated by means of curves of optical activity and of iodine titrations of mixtures of dehydroascorbic acid with each sulfhydryl compound (52).

The synthesis of *ll*-S-(β-amino-β-carboxyethyl)-homocysteine has been described (53). The very significant findings concerning the ability of this compound to support growth are described below. Brief reports have appeared concerning the synthesis of glycyl-*l*-methionine (54), guanido-methionine (55), and N-N′-diacetyl-sulfanilyl- and N-N′-disulfanilyl-*l*-cystine (56), *dl*-methionine sulfone (57), and the two α-amino-β-thiolbutyric acids corresponding to threonine and allothreonine (58) have also been synthesized. The synthesis and properties of the aspartic acid analogue of glutathione ("asparthione") have also been reported (59).

Occurrence of sulfur compounds in biological materials.—Methionine has been reported as completely absent from crystalline horse serum albumin (60). Cystine and cysteine accounted for all of the sulfur present. Similar results were reported by Ross (14) on the

protein of tobacco mosaic virus. The cystine and methionine content, as well as that of threonine and serine, of the purified peanut proteins, arachin and conarachin, have been reported (61) as have also the cystine and methionine content of proteins prepared from certain grasses (62). Cystine has been reported for crystalline pepsin in concentrations of 1.4 to 1.5 per cent (63). Progressive changes in the cystine content of hydrolysates of dog sera after infection with Type I pneumococcus were followed by both colorimetric and polarographic methods (64). The former gave uniformly higher results. The cystine content, paralleling the total nitrogen content, decreased during infection then returned to normal.

Using the criterion of growth, the cystine and methionine content of the proteins of certain molds was tested (65). Deficient growth of young rats, unless the diet was supplemented with cystine or methionine, led the authors to conclude that these mold proteins are low in both sulfur compounds.

New titration methods for determination of sulfhydryl content of proteins have been described (66), and one method, titration with ferricyanide, has been applied to egg albumin after treatment with various denaturants (67) and to surface films of the former (68). The films showed about the same degree of liberation of sulfhydryl groups as the denatured samples. Anson (69) has also studied the factors which influence the oxidation of sulfhydryl groups, chiefly by the Folin uric acid reagent. Figures for the sulfhydryl content of egg albumin have also been reported (70), using the reduction of free sulfur by means of the sulfhydryl groups and determination of the resulting hydrogen sulfide. The sulfhydryl content of serum albumin and also of milk has been reported (71).

The physical properties of wool fibers and their resistance to enzymes are reported as due to disulfide cross-linkages between polypeptide chains (72).

An interesting lipoprotein has been prepared from petroleum ether extracts of wheat flour. It is high in sulfhydryl content and probably serves as a natural activator of wheat proteinase (73).

Metabolism.—A comprehensive review of the metabolism of the sulfur-containing amino acids has been provided by Lewis (74). Tarver & Schmidt (2) have reported studies on the metabolism of methionine containing radioactive sulfur. This, fed to bile fistula dogs, produced taurine containing radioactive sulfur with smaller amounts of the latter found in other tissues. Oral administration of elementary

radioactive sulfur produced no radioactivity in any protein sulfur. Practically all radioactive sulfur recovered was in the sulfur fractions of the urine, chiefly sulfate. Recovery of the total amount fed was, however, very incomplete. Methionine containing radioactive sulfur fed to rats appeared about one third as urinary sulfate with the remainder distributed throughout the various tissues. About 14 per cent of that fed was found in the liver. Waelsch & Rittenberg (75) have followed the metabolism of glutathione by feeding glycine containing isotopic nitrogen to rats and rabbits. Analysis of tissues after periods two and one-half and seventy-two hours showed that the introduction of the glycine, so labelled, into glutathione was much more rapid than into general body protein although the absolute amount of the isotope in the protein was the greater. Of the isotopic nitrogen in glutathione, about two thirds was in the glycine component with the remaining one third distributed between the other two amino acids. A further report (76) describes the results of administration to rabbits of ammonia containing isotopic nitrogen and to rats of *dl*-glutamic acid, similarly labelled. In both cases after two and one-half hours, the glutathione of the liver and intestines contained appreciable amounts of the isotope. Previous conclusions as to the very rapid metabolism of glutathione are thus supported.

Addition of iodoacetic acid to a basal diet low in protein caused a statistically significant lowering of the sulfhydryl content of the tissues of rats (77). The loss was prevented by supplements of cystine. In further studies of the mercapturic acid synthesis in animals, Stekol (78) has demonstrated the synthesis of N-acetyl-S-*p*-bromobenzyl *l*-cysteine in the rat from *p*-bromobenzyl bromide, and S-*p*-bromobenzyl *l*-cysteine, or S-*p*-bromobenzyl glutathione. Acetylation *in vivo* of *p*-bromophenyl *dl*-cysteine by rats with excretion of the *d*-mercapturic acid has also been demonstrated (79).

In the acetylation of S-benzyl cysteine in rats and humans, the *d*-isomer is acetylated to a much lower degree than the *l*-isomer (80). The metabolism of *l*- and *dl*-α-hydroxy-β-benzylthiopropionic acid and *dl*-α-hydroxy-γ-benzylthiobutyric acid in the rat (81) and the mechanism of inhibition of growth by benzyl chloride have also been studied (82).

Thiourea, administered to rabbits, shows no evidence of any oxidation to sulfate, either inorganic or ethereal (83), nor could the small increases in the urinary output of cystine, reported by Medes (84) for man following thiourea administration, be demonstrated. The

thiourea was for the greater part excreted unchanged. S-carboxy methyl cysteine when administered subcutaneously to rabbits is not oxidized to sulfate. Oral administration produced slight increases in urinary sulfate, probably the result of decomposition by intestinal flora, and an ether-soluble sulfur compound giving a strong nitroprusside test for disulfide linkage (85). These results would seem to exclude for the rabbit the mechanism postulated by Brand and co-workers (86) for man, involving the formation of a disulfide compound containing the thioglycollic acid group.

A further development of the reports by Fromageot and co-workers (87, 88, 89) on an enzyme (desulfurase) in dog and rat liver, which converts cysteine and cystine to hydrogen sulfide, is the demonstration by Smythe (90) that such an enzyme is found in liver of a number of other species, including man. The other principal products from both cystine and cysteine were ammonia and pyruvic acid. Although carried out under anaerobic conditions, small amounts of free sulfur were produced, particularly from cystine. An apparent reversal of this reaction is also indicated (91) by the use of hydrogen sulfide containing radioactive sulfur. The latter was added, along with cysteine, to a preparation of the above desulfurase. When the nitrogen of part of the cysteine had been converted to ammonia, the remaining cysteine was isolated. It contained appreciable amounts of radioactive sulfur. In hog liver the reducing level set up by cysteine leads to more rapid autolysis. Two enzyme systems seem to be indicated, one activated by sulfhydryl compounds, the other not (92).

Employing diets with mixtures of purified amino acids, Rose & Wood (93) demonstrated that young rats synthesized from 4 to 6 mg. cystine per day. No significant differences in the cystine increments of different tissues were observed although in certain of their experiments only methionine could have furnished the necessary sulfur for cystine production. There is, as the authors carefully point out, no evidence that the carbon chain of the cystine originates in the carbon chain of the methionine.

Cystine with pantothenic acid is reported as markedly decreasing the time required for replacement of the gray hair of nutritional achromotrichia (94). Studies of the changes in mice after some months on a low cystine diet (95) and the delay in development of tumors from feeding *p*-dimethylaminoazobenzene at low cystine levels (96) have been reported.

Studies from the laboratory of Lewis (97) have provided figures

for the cystine content of plasma ultrafiltrates of normal bloods from the human, rat, rabbit, and hog. These values ranged from about 0.7 to 1.1 mg. per 100 ml. of the ultrafiltrate, regardless of species or fasting history. Methods for the determination of nonprotein sulfur and sulfate sulfur are also described (98). Administration of cystine by mouth raised its content in the ultrafiltrates to as high as 8 mg. per cent, and after subcutaneous injection, to nearly 17 mg. per cent. Administration of methionine by mouth also raised the cystine content to nearly 3 mg. per cent, and after subcutaneous injection of the methionine, to nearly 5 mg. per cent. These increases in the cystine content of plasma after methionine administration give further support to the hypothesis that cystine may be synthesized from the sulfur of methionine in the living animal. A similar conclusion (99) has been reached on the basis of the methods of Lafevre & Desgrez (24, 25).

Certain contributions have been made to the details of methionine metabolism. Deaminization by means of liver and kidney slices has been shown to produce the corresponding keto acid with about 2 per cent of the methionine going to sulfate (100). Nothing giving a nitroprusside reaction was formed, and no deaminization of either homocystine or homocysteine was observed. In confirmation of the results of Smythe (90) some production of hydrogen sulfide from homocysteine was observed. The feeding of methionine to experimental animals gives an increase in the keto acid fraction in the urine (101).

Continued studies by Virtue & Doster-Virtue on the mechanism of taurine production have shown that bile fistula dogs with a depleted taurine supply show an increased output of the latter when given methionine sulfoxide. However, a large share of the methionine sulfoxide appeared in the urine in the unoxidized (nonsulfate) fraction (102). Medes & Floyd (103) have announced an enzyme system which produces taurine from cystine and cysteine. They suggest, as the most plausible order of reaction, oxidation to the disulfoxide, decarboxylation of the latter, followed by oxidation to taurine.

A brief but interesting report by Blaschko (104) describes a decarboxylating enzyme from dog liver producing taurine from cysteic acid. Manometric measurements of carbon dioxide evolved gave one mole carbon dioxide per mole of *l*-cysteic acid and one-half mole carbon dioxide per mole of the *dl*-form. A further report (105) on enzymic oxidation of sulfur compounds suggests a route for the metabolic oxidation of cystine and cysteine. Besides cytochrome oxidase

and the enzymes that give rise to inorganic sulfate, others are described, among which is one which decarboxylates cystine.

A careful study has been made of the sulfur metabolism of a group of eight children ranging from eight to twelve years in age. Sulfur excretion and retention, urinary sulfur partition, nitrogen to sulfur ratios, etc. were determined (106).

Interesting and stimulating data are being afforded by various studies on the growth-promoting properties of certain of the thio ethers. The unsymmetrical thio ether, S-(β-amino-β-carboxyethyl)-homocysteine, the preparation of which was described by Brown & du Vigneaud (40), has been prepared as the *ll*-isomer, and the latter is found to be capable of substitution for cystine in the growth of young rats (53) if supplied with an otherwise insufficient quantity of methionine. However, added to a diet free from methionine, the thio ether did not support growth even on addition of choline. It would seem more likely, therefore, that this compound does not split *in vivo* to homocysteine but rather to cysteine. This finding gives promise of being a clue to the mode of conversion of methionine and homocystine to cystine *in vivo*. Of further interest is the report that cysteine is produced by the action of liver slices both on this mixed thio ether of cysteine and homocysteine (107) and on a mixture of homocysteine and serine (108), the presence of the latter being essential. The fact that methionine is an essential in the diet of the growing chick and that neither cystine nor homocystine alone can replace it without supplemental choline has been confirmed (109, 134), while on 18 per cent casein diets with added arginine and glycine, 0.3 per cent cystine was also needed for maximum growth (110). The arginine and glycine appeared just as essential as the cystine.

Further confirmation has been supplied that dietary methionine must be present in at least suboptimal amounts in order to permit partial substitution by cystine (111). In improving the growth-producing properties of peanut meal protein, methionine has been found to be effective and cystine not. Under these conditions both amino acids improved lactation, but the effect of methionine was the more marked (112). However, Sure (113) finds lactation of rats promoted by addition of cystine to an 18 per cent casein diet, indicating an insufficiency of methionine in this amount of casein.

In the diet of the rat, *dl*-methionine can be replaced by its keto acid (114). *dl*-Methionine methyl sulfonium chloride can also substitute for methionine whereas *dl*-methionine sulfone cannot (115). The

latter, however, did not appear to be toxic. A further report by Routh (116) on the nutritional status of powdered wool has shown that as the sole source of protein in an otherwise adequate diet, it is incapable of supporting the growth of young rats. Similar results were obtained with powdered chicken feathers, but the powdered wool was the better of the two (117).

Snell (118) has reported the preparation of the sodium and barium salts of N-α, γ-dihydroxy-β,β-dimethylbutryl taurine. He finds that these compounds inhibit the growth of various organisms requiring supplemental pantothenic acid. Addition of more of the latter reverses this effect. Methionine, cystine, and cysteine are effective in preventing toxicity effects of cobalt and nickel salts in young rats (119). On the other hand, a cobalt-cysteine complex prepared *in vitro* was found to be relatively nontoxic. It is suggested that the toxicity of these metals is the result of fixation and consequent loss of sulfhydryl compounds as mercaptides, thus causing interference with oxidative mechanisms. The relative insolubility of all heavy metal mercaptides should make this hypothesis, if correct, applicable to most or all cases of heavy metal poisoning.

The effect of thiol groups on the mechanism and activity of the lactogenic hormone has been studied (120, 121).

The metabolic connections between cystine and methionine and the relation these bear to problems of methylation and consequent fat metabolism still attract much attention. Since the relationships between various donators and acceptors of methyl groups in fat metabolism are discussed in another review, limited mention only will be made of some cases involving sulfur compounds. In its general form, the hypothesis, that liver damage results from too high proportion in the diet of those sulfur-containing amino acids unable to deliver methyl groups and unaccompanied by other potential sources of methyl groups, has been amply confirmed from various angles (122, 123). The transfer of methyl groups from methionine to choline and creatine has been demonstrated by feeding methionine with deuterium in the methyl group. Deuteriocholine and deuteriocreatine were isolated after such feeding and all deuterium was in the methyl group (124). Hemorrhagic degeneration in young rats is absent or slight if a low choline diet contains over 0.8 per cent methionine, regardless of how this level is attained. However, below this level of methionine, choline is a dietary essential, the need of which is greater with increased cystine (125). That the roles of methionine as a methylating

agent and as a purveyor of essential sulfur in a utilizable form are separate and distinct, is suggested by a report from Griffith's laboratory (126). Here the evidence indicates that methionine which is needed and used for methylation is not utilizable as a source of cystine sulfur. On an 18 per cent casein diet, supplementary cystine and methionine both improve the efficiency of food utilization. In absence of any dietary choline, however, about 30 per cent casein is required to prevent deposition of liver fat and hemorrhagic degeneration. On adding increasing amounts of cystine to a low-casein, high-lard diet, supplemented by methionine, no increase in liver fat was observed until very high levels of cystine were attained (127). It is suggested that the cirrhosis and the hemorrhage and necrosis resulting from feeding cystine to young rats are separate and distinct entities and that while choline and methionine tend to prevent hepatic cirrhosis, cystine and methionine prevent hemorrhage and necrosis (128, 129). A study of the effects of a number of different diets on hepatic lesions resulting from excess dietary cystine is similarly suggestive. Fatty infiltration occurred only with low-protein, high-fat diets (130). Even cysteic acid at 12.5 to 15 per cent levels has been reported as producing portal necrosis and cirrhosis of the liver within two weeks while *dl*-methionine at 6.4 and 12.4 per cent levels caused severe atrophy of liver cells but no cirrhosis. Similar amounts of taurine showed no such effects. The authors conclude that no relationship exists between necrosis or cirrhosis and the state of the sulfur in the compound (131). The prevention of dietary cirrhosis of the liver has also been studied by Blumberg and co-workers (132).

The subject is being further developed by tests for lipotropic activity on the part of a variety of other compounds. Cystine betaine, methionine sulfoxide, and dithiodiglycollic acid have shown lipotropic activity (133). Cysteine loses its effect in producing fatty livers when alkylated with various alkyl radicals and behaves more like methionine. Cystine disulfoxide behaves like cystine in this regard, whereas cysteic acid, as would be expected, has no influence. Other inert sulfur compounds are pentocystine, hexocystine, and djenkolic acid. In a similar study, Stelten & Grail (134) investigated both the quantity of liver lipids and their content of nitrogen, phosphorus, and choline at various levels of dietary choline with addition of such compounds as ethanolamine, serine, cystine, homocysteine, and guanidoacetic acid. Liver fat resulting from choline deficiency was expectedly low in lecithin. However, these investigators find that ad-

dition of cystine and homocysteine both raise the lecithin content. Much further work is obviously necessary before such seeming contradictions to other results can be explained. Ethanolamine caused no great rise in lecithin content but did raise the monoamino phosphatides. Guanidoacetic acid, as would be expected, caused a markedly fatty liver with a striking decrease in the choline content of the resulting fat. Earle & Kendall (135) showed that no connection whatever exists between hepatic lesions and sulfate production.

As a tool for use in the study of the relationships between choline and homocystine, Welch (136) has described a method of preparing a hydriodic acid hydrolysate of casein. The resulting demethylation of the methionine present and destruction of the tryptophane give a product highly suitable for feeding experiments. With this source of amino acids, the author has confirmed the conclusion that choline or betaine must be supplied with homocystine to prevent liver damage. On the other hand, oxidation of the sulfur of the methionine in casein to methionine sulfone, using hydrogen peroxide in formic acid solution, has given a product free from methionine (and tryptophane) (137). This modified casein, which requires supplementing with both of the above amino acids (138), gives promise of being a tool for carrying on methylation without supplying the necessary sulfur in available form.

It has been reported that methionine and to a less extent cystine protect protein-depleted dogs against injury resulting from chloroform poisoning (139). These very suggestive results were consistent whether the amino acids were administered by mouth or injected.

Cystinuria.—Lough and co-workers (140) have published a reinvestigation of the metabolism of cystine, cysteine, methionine, homocystine, and S-carboxy methyl cysteine in a twenty-seven-year-old female cystinuric from the point of view of the excretion of extra cystine. Administration of cysteine produced extra cystine excretion; cystine and homocystine did not. *dl*-Methionine produced no significant increase in cystine output except on a low level of protein in the diet (1.2 gm. protein per kilo). Under these conditions some extra cystine was obtained. Administration of large doses of S-carboxy methyl cysteine reduced the cystine excretion by about 25 per cent but did not produce the marked degree of reduction (about 80 per cent) reported by Brand and co-workers (86). The increase in cystine output resulting from diuresis reported by Andrews & Andrews (141) was not observed by these workers. Hess & Sullivan

(142) have reported on two cystinurics, one of which showed no increase in cystine excretion following large doses of methionine while the other showed such an increase. The latter also gave an increased cystine output after feeding certain nonsulfur-containing amino acids such as alanine, glycine, and glutamic acid. The effect of alanine was particularly marked. It seems obvious that uniformity of response between different cystinurics has yet to be attained.

The ingestion of methionine and cysteine by cystinuric dogs caused marked increases in cystine excretion at low levels of dietary casein content. At high levels of the latter the increase in cystine output decreased to within the limits of experimental error (143). Substitution of arachin for the casein produced less cystine excretion than did the corresponding levels of casein (144). Family trees of three more cystinuric families have been placed on record (145, 146). A series of reports on various clinical aspects of cystinuria has also been published (147, 148, 149, 150).

Arthritis.—A comparison has been made between serum proteins from normal and from arthritic subjects as regards total sulfur, total nitrogen, and amino acid content (151). No essential differences could be found and nothing indicated any connection between the arthritic condition and a disturbed sulfur metabolism (152). Furthermore, inconclusive results followed attempts to demonstrate clinical improvement by administration of colloidal sulfur in cases of rheumatic fever (153).

Insulin.—The feeling that an unstable sulfur group is an essential part of the insulin molecule has prompted tests on various sulfur compounds for hypoglycemic activity. Application of such tests to *bis, p*-amino benzoyl *l*-cystine have yielded negative results (154). Inactivation of insulin by injection of cysteine seemingly fails in adrenalectomized rabbits (155).

Selenium-sulfur relationships.—Selenium, in its relationship to sulfur only, will be discussed here. The isolation of a mixed sulfur-selenium compound (42) of the thio ether type has not only drawn attention to the properties of lanthionine but has stimulated further work on the toxicity of such selenium compounds. Comparisons of seleniferous wheat, selenium cystine, and sodium selenite showed the first two to have about the same toxicity. Arsenic was found to give full protection against the toxicity of selenium cystine (156). The protective action of arsenic is equally efficient whether applied as sodium arsenate or sodium arsenite, whereas the sulfides of arsenic

were ineffective. The protective action was equally effective against either sodium selenite or selenium cystine (157). Some results reported by Anderson & Moxon (158) leave open the question as to the possible formation of a selenium analogue of glutathione in the blood of selenized steers. Moxon & Schaefer (159) have reported attempts to separate cystine from its selenium analogue by dialysis. The latter is evidently less stable since loss of elementary selenium readily occurs.

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CARBOHYDRATE METABOLISM

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Absorption

Under conditions of voluntary feeding it has become apparent from the work of MacKay & Clark (1), first reported last year, that the rate of absorption of glucose under certain conditions may be considerably higher than the generally accepted value of 100 mg. per 100 sq. cm. of body surface per hour. This figure had been obtained by administration of concentrated glucose solution by stomach tube. Values as high as 300 were noted in rats injected with protamine-zinc-insulin or in the animals in which metabolism was increased by subjection to cold. In depancreatized rats Pauls & Drury (2) now report absorption coefficients varying from 270 to 470 mg. per 100 sq. cm. per hour although the fluctuation in values in thirteen of the fifteen rats was only between 312 and 358 mg. These authors point out that if an absorption coefficient of 100 were constantly present, the amount of carbohydrate which could be absorbed would account only for the minimum basal requirements. Since the above values were obtained by calculation of glucose solution consumed during the twenty-four hours, it is probable that higher values may obtain for limited periods during high alimentation. Pauls & Drury (2) believe that the absorption coefficients are not necessarily high as a result of the need for increased amounts of glucose, but because

> the absorption mechanism of the intestinal tract operates most efficiently under conditions governed by many functions such as gastric motility, secretions of various parts of the tract, etc., and the disturbance of these, which is usually produced by forced feeding leads to gross inefficiency and low absorption coefficients.

The above experiments also demonstrate that it is operative under conditions where a hyperglycemia as well as a hypoglycemia obtains. One is now practically forced to the conclusion that the rate of absorption is not constant but varies with a number of factors. It would be of interest to compare the rate of absorption of fructose and of galactose where the feeding is voluntary and when the animal is subjected to similar conditions. Pierce, Haege & Fenton (3) believe that the

rate of glucose absorption in dogs is dependent on the size of the meal. Dilution invariably occurs in the stomach when 50 per cent glucose solutions are given although some dilution may occur in the duodenum. Driver (4) also has found that the rate of glucose absorption decreases with time but he questions whether the usual method for such measurement is a sufficiently normal physiological one to place much value on the data obtained.

Further evidence on the role of the adrenal glands in the absorption of glucose is to be found in the results of Clark & MacKay (5), who found a decreased rate in rats where the salt balance was upset, in confirmation of the results of Cori & Cori (6) and Verzar & McDougall (7). However, when the normal salt balance was preserved, the absorption of glucose was not diminished in operated rats in confirmation of the earlier report of Deuel *et al.* (8) and of Althausen, Anderson & Stockholm (9). This would seem to offer the necessary proof that although the role of the adrenal may be of great importance in glucose absorption, it is only an indirect one and acts by maintaining a normal salt balance, thus preventing hemoconcentration and the reduction in blood flow to the gut, leading secondarily to a slowing up of absorption.

The assumption that phosphorylation of glucose takes place during absorption gains some further support from the experiments of Beck (10) in which an increase in organic nonpyrophosphate (presumably hexose phosphate) was found in rat intestinal mucosa when glucose was being absorbed. Although this increase was less than 0.5 per cent of that calculated for the quantity formed when 0.25 gm. of glucose is transformed to hexose phosphate, it is suggested that because of the rapid turnover due to the speed of phosphorylation and dephosphorylation a larger accumulation is not to be expected. The quantity of "hexose phosphate" was decreased after administration of phlorhizin.

Golden & Long (11) have employed the Cori technique to follow the absorption of glucose in the chick. They found a constant rate up to four hours of 400 mg. per 100 gm. per hour (which would be 224 mg. per 100 sq. cm. per hour). After this period the liver glycogen had increased to 6 per cent and the muscle glycogen to 1300 mg. per cent, but these accounted for only 20 per cent of the total absorbed. It was concluded that either the chicken possesses an intense ability to oxidize glucose or it is remarkably efficient in converting it to fat. Indirect estimations of the rate of absorption in man were carried

out by determining the rate of development of hyperglycemia [Goldfarb & Golden (12)]. This initial rise in blood sugar was most rapid when the glucose was given in a 5 per cent solution and became progressively slower with 10, 15, and 30 per cent concentrations. In addition the most dilute sugar was most effective in the arousal of patients from insulin shock. These authors believe that glucose absorption is at least partially dependent on the establishment of an equilibrium between the glucose in the intestine and in the blood serum.

The increased galactosemia in thyrotoxicosis is apparently caused both by the increased rate of absorption of galactose from the intestine as well as by the decreased rate of liver glycogenesis [Grauer, Starkey & Saier (13)]. A similar condition gradually develops when thyroxine is administered for a period of sixteen to twenty days. The same workers also report that stilbestrol increases the rate of absorption of galactose as well as glycogenesis. Galacturonic acid, on the other hand, is absorbed to less than 10 per cent from the intestinal tract of a dog or man [Werch & Ivy (14)].

Glycogenesis

Colowick & Sutherland (15) have demonstrated the synthesis of polysaccharide from glucose when purified enzymes are employed. Purified hexokinase converts glucose to glucose-6-phosphate; the energy for this reaction is largely supplied by adenosinetriphosphate. When phosphoglucomutase of high purity was employed either with glucose-6-phosphate or glucose-1-phosphate, the equilibrium mixture contained approximately 95 and 5 per cents, respectively, of the above components. The equilibrium was unaffected by pH and only slightly by temperature. When purified phosphorylase was also used, a rapid synthesis of polysaccharide resulted. In the presence of phosphorylase 77 per cent of polysaccharide was at equilibrium with 23 per cent of glucose-1-phosphate. In muscular atrophy a decreased rate of phosphorylysis was found to be associated with a decrease of phosphoglucomutase content without change in phosphorylase (16). A somewhat different mechanism obtains in the synthesis of starch. Green & Stumpf (17) have separated a highly active phosphorylase from the potato. This enzyme was demonstrated earlier in peas and potatoes by Hanes (18). The enzyme differs from animal phosphorylase in not containing adenylic acid. As might be expected, only glucose-1-phosphate is active with the enzyme. However, to insure a synthesis of starch, a third component is necessary in addition to the enzyme and

substrate. This is a catalytic amount of a polysaccharide such as glycogen, starch, or dextrin. To be catalytically active such a polysaccharide must be composed of α-glucose units in 1,4-glucoside linkage and be capable of being degraded to glucose-1-phosphate in the presence of inorganic phosphate and the enzyme. Polysaccharides having six to twelve saccharide components were effective but *alpha* and *beta* dextrins with four to six units were inactive. Another phosphorylase was also prepared from lima beans which apparently did not require the polysaccharide as a catalytic agent. Although Green & Stumpf (17) consider that this catalytic substance may be present as a component of the lima bean enzyme, they postulate that this enzyme system may be an entirely different one. Meyer & Bernfeld (19) have found that potato phosphorylase hydrolyzes only those complexes having 1,4-glucosidic linkages while yeast phosphorylase apparently is composed of two enzymes, one of which also acts on 1,6-glucosidic linkages (20).

The view that maltose is the sole end product of the diastatic reactions on starch is challenged by Stark (21) who believes that both maltose and glucose are formed as well as nonfermentable dextrins. It is indicated that no direct conversion of maltose to glucose takes place.

A crystalline muscle phosphorylase has been prepared from rabbit muscle by Green, Cori & Cori (22) which crystallizes as an adenylic acid complex, the latter prosthetic group being necessary for its activity. Here also it is noted that a catalytic trace of glycogen is required for its action. Cori & Cori (23) have reviewed the subject of enzymatic synthesis of polysaccharide completely in an earlier article. Phosphorylase is also present in the epiphyses of growing rats and rabbits where it may play a significant role in the calcification of cartilage by furnishing the glucose-1-phosphate essential as a substrate for phosphatase (24). Phlorhizin and phloretin which inhibit glycogenolysis also stop calcification. The fact that calcification can be restored by the addition of the Cori ester indicates that the failure in synthesis of this compound is the limiting factor.

Vennesland *et al.* (25) also interpret their results as indicating that the pathway for the synthesis of glycogen *in vivo* is through glucose-1-phosphate. Although when radioactive bicarbonate was injected into fasting rats which were fed glucose, the liver glycogen formed contained 13 per cent of C^{11} atoms, much lower amounts of isotope were incorporated in the polysaccharide when the synthesis

occurred *in vitro* with rabbit liver slices. In the latter case it is assumed that much less glucose is first broken down to pyruvic acid which is able to incorporate the isotopic carbon dioxide; a larger proportion must first be transformed directly to glycogen. This would indicate that the formation of trioses is not a necessary step in the conversion of glucose to glycogen. However, these data do suggest that a constant equilibrium is present in the various components of the carbohydrate system which results in a distribution of labeled carbon atoms throughout when introduced at any one stage although there may be no net formation of glycogen from precursors containing three or four carbon atoms. It is postulated that such interchange can occur if the substance can be brought into enzymatic equilibrium with pyruvic acid. A similar proportion of the glycogen (12 per cent) derived from the (+4) C atom in *in vivo* and *in vitro* studies with pyruvic acid and isotopic potassium bicarbonate was demonstrated by Buchanan *et al.* (26).

Two additional amino acids, i.e., histidine and valine, which Dakin (27) did not consider to be glucose formers, have now been shown to be glycogenic. Remmert & Butts (28) found that *l*(−)-histidine gave liver glycogen and lowered an endogenous ketonuria in rats although a hematuria resulted and a lag in glycogenesis obtained. These results were confirmed by Featherstone & Berg (29) who in addition showed that *d*(+)-histidine was only slightly glycogenic. These authors did not note any lag in the formation of glycogen from the *l*(−)-isomer. Since the rate of glycogenesis was similar when *l*(+)-glutamic acid was fed in amounts equivalent to the *l*(−)-histidine retained, it is suggested that the former compound may be formed as an intermediate. Such a pathway of metabolism is rendered more probable since it is known that the enzyme, histidase, can bring about such a change (30). Rose, Johnson & Haines (31) have demonstrated that *l*(+)-valine, *d*(−)-valine, and *dl*-valine as well as α-keto-isovaleric acid contribute three of the five carbon atoms to glucose in phlorhizinized dogs. This supports the recent work of Butts & Sinnhuber (32) who found some glycogenesis and a lowered ketonuria following the administration of *l*(+)-valine. Sharp & Berg (33) have re-examined the fate of lysine and obtained no evidence of glycogenesis or ketonuria although glutaric acid which is considered as a possible intermediate was ketolytic. The fate of tryptophane remains somewhat obscure. Although Borchers, Berg & Whitman (34) found that *l*(−)-, *d*(+)-, and *dl*-tryptophane as well as *l*(−)-kynurenine

and indolepyruvic acid markedly reduced an exogenous ketonuria produced by the administration of sodium butyrate, no glycogenic action could be demonstrated in the liver after feeding any of these substances. These results can be interpreted as evidence of the glycogenic ability of tryptophane and related compounds only if a ketolytic effect is caused by amounts of glucose too small to induce glycogen storage. That this might be the case is indicated by the results of Deuel *et al.* (35) which demonstrated that the administration twice daily of 50 mg. of glucose per 100 sq. cm. of body surface was sufficient to reduce endogenous ketonuria in the rat. This quantity of glucose must certainly be without marked effect on the liver glycogen.

Although the reviewer has felt that a proportionality usually exists between the ability of a substance to lower an exogenous or endogenous ketonuria and its efficiency in causing glycogenesis in the liver (35), it seems evident that this viewpoint must be somewhat modified. Borchers, Berg & Whitman (34) have suggested that the production of ammonia and formation of urea may result in some decrease in ketonuria inasmuch as the administration of ammonium chloride had such an effect; however, these authors also point out that since the ketolytic activity of indolepyruvic acid which contains only indole nitrogen is the same as tryptophane, other factors must be involved. Possibly muscle glycogen should be used as the criterion of proportionality rather than liver glycogen. Johnston & Deuel (36) have found that, although glucose lowers exogenous ketonuria twice as much as sorbitol and more than four times as much as mannitol or dulcitol, sorbitol invariably produces a greater deposition of liver glycogen than glucose when both substances are introduced intraperitoneally. On the other hand, the administration of glucose is immediately followed by a rise in muscle glycogen to a maximum level in six hours while only a small effect results after this interval from sorbitol. Muscle glycogen continues to rise after administration of sorbitol so that an identical value with that of glucose obtains at eighteen hours; this level is considerably lower than the maximum reached in six hours in the glucose tests. Tuerkischer & Wertheimer (37) describe adipose-tissue glycogen which is similar in all physical and chemical respects to liver glycogen. It is only present under certain well-defined conditions, that is, when a high carbohydrate diet is fed after a period of starvation. It reaches a maximum level as high as 1 per cent in four days after which it decreases and may disappear.

Liver glycogen is decreased to about the same extent after ether anesthesia whether the previous diet had been carbohydrate or protein; however, Newburger & Brown (38) found a much more rapid decrease in liver glycogen when the animals previously on the carbohydrate diet were subjected to fasting. These results were considered to be confirmatory of those of Mirski *et al.* (39). A diurnal rhythm in amount of liver glycogen in white mice has been reported by van Weel (40). This rhythm occurs both in fasted and fed animals. The maximum values obtain in the morning and the minimum values in the evening from 5 to 8 P.M. Apparently there is nothing to indicate that this is not the result of the nocturnal feeding habits of the mouse inasmuch as this has been shown to be the reason for this phenomenon in the white rat [Deuel *et al.* (41)]. The level of liver glycogen is also related to the strain of white rats. Orten & Sayers (42) report that the level in the Yale rats is always lower than in the Wistar strain after glucose injection, which they interpret as related to a disfunction of the anterior pituitary gland in the former group. The level of liver glycogen was found to vary with species and age; in general, glycogenolysis is more rapid in old animals than in young ones (43). The glycogen content of the endometrium has been shown to undergo cyclic changes, being lowest in the proliferative stage and highest in the differentiative phase [Randall & Power (44); Spyker & Fidler (45)]. In thiamin-deficient rats Harper (46) noted a slower rate of glycogenesis from glucose to be associated with a slower absorption of glucose although the animals were able to transform *l*(+)-sodium lactate or sodium pyruvate into hepatic glycogen normally. The liver glycogen of fed chicks was shown by Golden & Long (47) to average about 3 per cent. The level of glycogen in the pectoral muscle was 1000 mg. per cent and was found to be three to four times that of the gastrocnemius.

Blood Sugar

The mechanism of the hyperglycemia following anoxia in dogs has been further elucidated by McQuarrie, Ziegler & Hay (48). These authors believe that hyperglycemia results from glycogenolysis due to adrenaline produced by sympathetic adrenal stimulation. Not only is the hyperglycemia abolished after adrenalectomy in dogs, but the resultant hypoglycemia is attributed to the stimulation of the vago-insulin mechanism since anoxia also stimulates the parasympathetic system.

Carbohydrate tolerance is lowered in partially depancreatized dogs following prolonged administration of protamine-zinc-insulin to the extent that hyperglycemia, glycosuria, and ketonuria occur when the insulin is discontinued. This is explained by Mirsky *et al.* (49) as a "disuse atrophy." Fatty infiltration of the liver also may lower glucose tolerance. Treadwell *et al.* (50) found a marked lowering in tolerance in rats following the administration of a high fat, low lipotropic diet. This result was confirmed by Deuel & Davis (51) who also noted that the effect was more severe on female rats. No direct relationship between glycogen and fat level of the liver and extent of the increased response of blood sugar was found by the former investigators; the deposition of liver glycogen and lipid progresses independently. A permanent diabetic tolerance curve was produced in rabbits by massive doses of *Staphylococcus aureus* or its toxin [Nicholson & Holman (52)]. Possibly the low ascorbic acid content in the blood during infection is the cause of the lowered glucose tolerance, and it has been shown by Secher (53) that tolerance curves of the diabetic type can be altered by administration of ascorbic acid. Sulfanilamide was found to be without effect on general level of blood sugar or glycogen storage, but sulfapyridine and sulfathiazole both caused an increase in blood sugar although the former was more toxic as shown by the greater glycogenolysis in the liver (54). Fasting blood sugar values were within normal limits in aged subjects, but the response of blood sugar to glucose feeding is slower and the return to the fasting level more retarded although there is no evidence of reduced pancreatic function (55). The fasting blood sugar of chicks is about twice that of mammals, being about 200 mg. per cent, but this could be readily reduced by small doses of insulin to hypoglycemic levels (47).

Intermediary Reactions and Oxidation

The relationship of lactate and pyruvate to each other as well as to carbohydrate metabolism has been considerably amplified during the past year. It is now generally agreed that pyruvic acid is not only the direct precursor of lactic acid but also the substance immediately preceding the further degradation of the 3-carbon intermediates. Insulin is apparently necessary for its formation. Although it increases in the blood of normal animals on the administration of glucose (56), Bueding, Fazekas, Herrlich & Himwich (57) found that this did not occur in depancreatized dogs unless insulin was given and that the response was increased if glucose was administered simultaneously

with insulin. They also report that a similar condition obtains in diabetic patients. The latter finding has been confirmed by Klein (58) who found normal pyruvate and lactate in practically all subjects but no rise in these components occurred when insulin or glucose was given alone. Both lactic and pyruvic acids were significantly increased when glucose and insulin were given together and the rise occurred with the increased utilization of glucose. This conception is somewhat difficult to understand in view of the report of Shorr (57a) who found that lactic acid can still be produced by diabetic muscle from depancreatized dogs although its oxidative mechanism may be entirely lost.

Insulin is not required for the removal of pyruvic acid from the blood (57) in confirmation of the earlier report of Flock, Bollman & Mann (59) although there is no proof that oxidation has occurred. Although it has been repeatedly confirmed that the decarboxylation of pyruvic acid and its further degradation requires the presence of cocarboxylase, thiamin-deficient rats are able to form normal amounts of liver glycogen after administration of sodium *d*(—)-lactate or sodium pyruvate (46).

Stotz & Bessey (60) have noted a remarkably uniform relationship between blood lactate and blood pyruvate in men, rats, and pigeons when sufficient thiamin is present. Such factors as exercise, excitement, carbohydrate ingestion, and anoxia, which for a long time have been known to cause a rise in blood lactate, bring about a parallel increase in blood pyruvate although it may require five to six minutes for the equilibrium to be reached after mild exercise (61). The equations expressing the relationship for man are the following:

Less than 20 mg. lactic acid:

$$\text{Blood pyruvic acid} = (\text{blood lactic acid}) / 12.2 \qquad (1)$$

More than 20 mg. lactic acid:

$$\text{Blood pyruvic acid} = 1.05 - 0.0264\ (\text{blood lactic acid}) \qquad (2)$$

In rats, equation (1) is modified by changing the divisor to 9.44 while in equation (2) the constant employed is 1.60 and the factor by which the lactic acid is multiplied is 0.026.

In thiamin-deficient pigeons the equilibrium is upset due to the accumulation of pyruvic acid (P.A.). The severity of the avitaminosis may be judged by the value for the $\text{P.A.}_{\text{excess}}$. This is calculated by the above equations as follows:

$$\text{P.A.}_{\text{excess}} = \text{P.A.}_{\text{found}} - \text{P.A.}_{\text{calc.}}$$

In normal birds the limits of $P.A._{excess}$ were between -0.14 and $+0.21$. A progressive rise occurred on thiamin-deficient diets; values as high as 7.36 were obtained in birds with opisthotonus. These values were promptly lowered on administration of thiamin. The calculation of $P.A._{excess}$ makes it possible to recognize a relatively mild thiamin deficiency.

Not only was a hyperpyruvemia found both in the blood and spinal fluid of patients where a definite thiamin deficiency occurred, but Bueding, Wortis & Stern (62) also report a similar condition in twenty-two cases where fever was present. These authors suggest that this may be the result of thiamin deficiency occasioned by the high requirement caused by the elevated metabolism. However, Pilgrim *et al.* (63) have reported that in addition to thiamin, pantothenic acid and biotin each are important in rats to insure the removal of pyruvic acid from the blood. It has been found that the fasting pyruvic acid value may not be indicative of an altered metabolism but frequently the hyperpyruvemia only becomes apparent following glucose ingestion (62).

It has been well known for some time that the oxidation of alcohol is accelerated by the simultaneous metabolism of carbohydrate. This synergistic effect has been shown by Westerfeld, Stotz & Berg (64) to be caused by pyruvic acid. The rate of disappearance of alcohol from the blood of dogs was increased 260 per cent by the oral administration of pyruvic acid. Presumably the alcohol is converted to acetaldehyde by alcohol dehydrogenase in the presence of niacin and this condenses with pyruvic acid to acetoin (65). The frequent association of polyneuritis and pellagra in chronic alcoholism thus has a rational explanation.

The mechanism for the conversion of lactic acid to glycogen is further explained by the experiments of Vennesland *et al.* (66) with lactic-acid–containing radioactive carbon in the α and β positions. In distinction to the earlier experiments (67) in which glycogen containing only 1.6 per cent radioactive carbon was deposited after administration of Type I lactate (C^{11} in carboxyl group), the glycogen after administration of Type II lactate (C^{11} in *alpha* or *beta* position) contained twice the amount of the C^{11}. This is explained by the hypothesis that pyruvic acid formed from lactic acid enters the Kreb's cycle forming the dicarboxylic acids; before transformation to glycogen one of the carboxyl groups is lost and in 50 per cent of the instances it is the tagged carbon. When Type II lactic acid is used, the carbon iso-

tope is not lost by decarboxylation so that the full quota is found in the synthesized glycogen.

The combustion of glucose was no greater in normal men after the intravenous injection of 50 gm. of glucose than after its oral administration (68). Conn *et al.* (69) dispute the earlier claim of Soskin & Levine (70) that the utilization of sugar by the eviscerated dog is directly proportional to the blood sugar by showing no increase in carbohydrate combustion in normal fasting men when the blood sugar is elevated by epinephrine, provided that the experimental periods were long enough to allow acid-base balance to be restored. Moreover, the amount of glucose oxidized after its oral administration was the same with or without epinephrine with the consequent widely divergent levels of blood sugar. At a given hyperglycemia level, approximately twice as much glucose was found to be consumed if the elevated blood sugar was produced by the ingestion of glucose as when it was caused by the injection of epinephrine. On the other hand, Greeley, Martin & Hallman (71) report that more glucose disappears at high blood sugar levels in diabetic patients, depancreatized dogs, and rabbits under the action of insulin than at the normal physiological level. Pyruvate is readily utilizable by brain suspensions (72) and it is used in preference to glucose. Lactate is less readily metabolized by normal brain than glucose or pyruvate but it can account for the entire respiration in brains from insulinized animals. Other carbohydrates as well as acetic acid were nonutilizable. Writhington (73) found that neither glucose nor sucrose altered the muscular efficiency and that more glucose was used than sucrose.

Reinecke (74) could account for less than one half of the fructose injected into eviscerated rats which would indicate that it can be used in the peripheral tissues. Rynbergen, Chambers & Blatherwick (75) found that the initial rise in R.Q. after fructose ingestion in normal subjects was absent in fructosuric individuals. They believe that there is diminished oxidation or a failure of blood lactate to increase. Sachs, Sternfeld & Kraus (76) also noted the absence of increased blood lactate in patients suffering from fructosuria when fed fructose.

Endocrine Factors

Pituitary.—Working with eviscerated rats, Russell (77) showed that a greater drop in blood sugar occurred in the hypophysectomized animals than obtains in animals with an intact pituitary. Such an ac-

celerated removal of glucose from the blood could not be explained by an augmented muscle glycogen or lactic acid; the author presumed that it was due to increased oxidative utilization of carbohydrate in the peripheral tissues in the absence of the pituitary hormones. Unfortunately, it is impossible to confirm the increased combustion of carbohydrate by studies on respiratory quotients because the experiments are necessarily of too short a duration to obtain reliable values. Another possible explanation for the increased rate of glucose utilization might also be a more rapid change to fat. The interpretations of Russell (77) are contradictory to those of Soskin, Levine & Lehman (78), which she explains are due to the method of calculation but support those of Greeley (79). On the other hand, de Bodo *et al.* (80) believe that Russell's results cannot be explained solely on the basis of an increased utilization. They point out that in earlier experiments [de Bodo, Barker & Benaglia (81)] glucose utilization was quadrupled without a lowering in blood sugar while in the experiments of Russell (77) the rate of disappearance was only doubled. These authors consider that the production of glucose not only from noncarbohydrate sources but even from liver glycogen must be deficient after hypophysectomy, for they report results of low blood sugar in operated dogs in spite of normal liver glycogen. A possible explanation for this phenomenon is to be found in the report of de Bodo, Bloch & Gross (82) in which it is demonstrated that intravenously injected adrenaline produces only slight hyperglycemia in hypophysectomized dogs compared with normal dogs having a smaller content of liver glycogen. In neurohypophysectomized dogs a normal response was obtained. These experiments answer the criticism that failure in absorption of subcutaneously injected adrenaline in dogs after pituitary removal is the explanation for the divergence from normal. Samuels, Reinecke & Ball (83) believe that the fundamental disturbance after hypophysectomy is in the mobilization of endogenous stores of protein and fat. Operated rats previously maintained on a diet containing 90 per cent of their foodstuff as fat were found to maintain their blood sugar levels better than animals previously receiving a high carbohydrate diet. The liver glycogen was less readily broken down during fasting when the rats had been on a high fat diet (84). In another report these authors also suggest that there may be an increased rate of conversion of carbohydrate to fat (85). Herring, Fraenkel-Conrat & Evans (86) found that neither thyroxine nor the thyrotropic hormone of the pituitary was able to prevent the develop-

ment of hypoglycemia in hypophysectomized rats, a result which is contrary to those of Soskin *et al.* (86a) on hypophysectomized dogs. The rate of removal of intravenously injected fructose from the blood is slightly decreased after hypophysectomy (87). Injection of a saline extract of the anterior pituitary was followed by a diabetic glucose tolerance curve in adult rats but this moderated on repeated injections; in young rats the only response was an increase in glucose tolerance (88).

Adrenal cortex.—The role of the adrenal cortex in phosphorylation of carbohydrate was further explored by Verzar & Montigel (89). In minced muscles from normal rats, a rapid phosphorylation of glycogen occurs with a concomitant decrease in inorganic phosphate. However, when the muscle mince is prepared from previously adrenalectomized rats all in good nutritional condition, a marked reduction in the rate of phosphorylation obtains; *in vitro,* this value could be restored to normal by desoxycorticosterone. However, the absence of glycosuria in adrenalectomized rats would seem to indicate that the depression in phosphorylation is not sufficiently generalized to prevent the resorption of glucose in the kidney from the glomerular filtrate, a phenomenon almost certainly connected with phosphorylation. In this experimental procedure the criticism offered on the effect of the adrenal cortex in the intestine (8) is not valid. Reinecke & Kendall (90) have suggested a new method for the bioassay of the cortical hormone based on the extent of increase in liver glycogen of adrenalectomized male rats fasted one day and previously maintained on 1 per cent saline. In fasted chicks adrenal cortical extracts raise liver glycogen but no further elevation attends the injection of this hormone in fed birds (47).

Sex glands.—The higher glycogen values found earlier in fed and fasted male rats as compared with female rats (91) is confirmed by Grayman *et al.* (92). These authors consider this to be the result of a greater speed in glycogenolysis, possibly due to a more rapid fat synthesis, since glycogenesis is approximately the same in both sexes. Apparently the lower level of oxidation in the female precludes the greater rate of destruction as the cause. After castration of the male, or female, the results were similar to normal female rats although the injection of testosterone did not alter the response of female rats. It is suggested that the female rat either stores glycogen in the muscles to a greater degree or converts carbohydrate to fat at a greater rate than the male. This is because the testes produce some factor which in-

hibits these processes. The above interpretations are difficult to reconcile with the results of Gulick *et al.* (93), which showed that the sex differences in glycogen were abolished by ovariectomy, and the results of Grunewald *et al.* (94), which demonstrated that the sex variations in exogenous ketonuria also disappeared after removal of the ovaries. There are, therefore, two interpretations of the difference, viz., that the female possesses a hormone accelerating the disappearance of carbohydrate, and that the male retards the breakdown of carbohydrate by a specific hormone. Further experiments are necessary to establish which of these conceptions is correct. Deuel & Davis (51) have shown that the glucose tolerance of female rats on a low protein, high fat diet, low in lipotrophic factors, is lowered to a greater extent than the male rats. Deane (95) has found that a sex difference in cell structure of the liver occurs which is associated with a greater swelling of mitochondria and a more persistent accumulation of fat in the females. Liebson & Liebson (96) found variations in the hyperglycemia in bitches, induced by the feeding of sucrose, concurring with the estrus cycle; the highest values occurred at the second half of anestrus whereas an incipient fall occurred at the beginning of proestrus. This would suggest that the entrance of estrone coincided with a more rapid disposition of administered sugar. No effect was noted on the fasting blood sugar value. The marked hypoglycemia following hypophysectomy in guinea pigs is exaggerated by the injection of estrone or estradiol benzoate although there is no effect on liver glycogen. No change in blood sugar occurred in normal guinea pigs after injection of the estrogens (97). Janes & Nelson (98) found that repeated injections of stilbestrol tend to increase blood sugar; liver glycogen was increased after a shorter period of administration and the effect was greater on males than females and more pronounced in the castrates than in the normal rats. In a later report (99) they conclude that the effect of stilbestrol on liver glycogen is caused by the cortical hormone secreted as a result of the release of adrenotrophic pituitary hormone.

Pancreas.—The effect of administration of insulin on the glycogen content of liver and muscle of fasted depancreatized rats was investigated by Drury & Pauls (100) who found a marked augmentation in muscle glycogen (0.16 to 0.60 per cent) and also in liver glycogen (3.26 to 9.78 per cent), which is in harmony with the earlier work of Cori & Cori (101). However, these increases account for only about one quarter of the sugar which disappears. These authors believe that the best explanation is that insulin stimulates a conversion

of glucose to fat. The other possible metabolic fates would be oxidation (which is precluded by the increased metabolic rate required), conversion to protein (which could account for only 8 per cent), or storage as some nonprecipitable compound as hexose-1-phosphate. The last would entail the utilization of larger amounts of phosphate than are available. Richter (102) has shown that insulinized normal rats have a markedly increased appetite for glucose solution which is quite specific inasmuch as the consumption of stock diet is not greatly increased.

Vitamins

The administration of thiamin to rats on a diet containing only glucose markedly prolongs the length of life. Richter & Rice (103) found that rats survived on a diet of glucose for thirty-six days as compared with four days for fasting with water; however, when thiamin was also added, the survival was more than double (seventy-four days) and the rats were quite active for as long as fifty days. Bollman (104) was able to increase the survival time of young rats from two to as long as sixteen days by the simple expedient of adding thiamin to the diet. With the possible exception of niacin, the other members of the vitamin B complex were inactive. The beneficial effect of adding yeast to the diets of thyroid-fed rats is to be traced to the improved appetite with the resulting increased food intake since the rats receiving thyroid have a higher requirement for thiamin (105). Pantothenic acid in the blood of rabbits is markedly lowered by glucose administration although it is possible the results might be partly explained by blood dilution (106).

Carbohydrate and Ketone-Body Metabolism

The mechanism by which the metabolism of carbohydrate causes a lowering in ketonuria is still far from satisfactorily answered. Somogyi (107) has suggested that the experimental evidence has completely invalidated "ketolysis" as an explanation for such activity. He postulates that since carbohydrate is the preferential source of energy of the liver, ketone bodies are produced in increased amounts only when liver glycogen is exhausted and fat must be used as a source of energy in that organ; when the hyperketonemia so produced exceeds the utilization of ketone bodies by peripheral tissues, ketonuria obtains. In a later report Somogyi & Weichselbaum (108) describe increases in ketonemia in two diabetic subjects after sugar adminis-

tration although a decrease occurred in the third. This is explained by the fact that although increased blood sugar may cause glycogenesis in the liver, this organ cannot utilize carbohydrate, and ketone bodies continue to be produced as a source of energy; in the muscles, on the other hand, the carbohydrate is used in preference to the ketones with the resultant increase of ketones in the blood. Somogyi's theory is an extension of the antiketogenesis hypothesis.

However, it is difficult to interpret the experiments of Bobbitt & Deuel (109) on the antiketogenesis theory. These authors demonstrated that the quantity of ketone bodies recoverable from rat liver slices immersed in a medium containing butyrate was always less if glycogen also had been added; in addition, the amount of butyrate which disappeared was also always greater. There was an increased rate of removal of the ketone bodies to the extent of about 20 per cent when glycogen was present. If glucose is the preferred substrate, then either the butyric acid should not have been metabolized to ketone bodies when glycogen is present, or if oxidized to ketone bodies they should accumulate and be quantitatively recoverable. Butyric acid probably could not have been converted to a nonrecoverable substance, such as a long-chain fatty acid; there is no evidence that long-chain fatty acids containing deuterium are present after feeding deuterotributyrin (110). The fact that glycogen (which is nonpermeable) is active while glucose (which is permeable to cell membranes) is inactive is difficult to explain. Possibly, hexose phosphate is the ketolytically active intermediate and it cannot be formed from glucose by the liver slice but can be from glycogen. The difficulty in phosphorylation of glucose may account for the failure of glucose to be changed to glycogen in liver slices.

It is also difficult to interpret the experiments demonstrating the effect of glucose on exogenous ketonuria by the theory proposed by Somogyi. In these experiments it was demonstrated that an amount of glucose as small as 50 mg. (35) is able to lower ketonuria. When the animal is maintained on a stock diet, the ketone bodies are practically entirely absent from the urine in spite of the fact that acetoacetate (111) or β-hydroxybutyrate (112) was given in large amount. A typical experiment of Butts (111) showed values of 3 mg. per day excreted when acetoacetate was fed while the rat was allowed access to food. This value rose to 63 mg. when the food was removed and the acetoacetate continued. If no ketones were being produced by the liver or oxidized by the peripheral tissues due to the preferential

oxidation of carbohydrate, then all the administered acetoacetate should have been quantitatively recoverable when the food was fed. Another fact unexplained by the Somogyi theory, is the failure of alcohol to lower ketonuria to the same extent as glucose (35). Alcohol is able to replace fat oxidation and for this reason should be antiketogenic.

Somogyi (113) reported an increased ketonemia associated with various conditions causing hypoglycemia, which is believed to be related to an increased rate of hepatic glycogenolysis. Fasting ketosis in the rat was found to be related to the nature of the protein ingested during the preceding period (114). The ketosis was greatest after including gelatin in the diet preceding fasting, intermediate after adding casein and edestin, and least after adding blood albumen. Shaw (115) found that β-hydroxybutyric acid was used by the lactating mammary gland of the cow; 37 per cent of the oxygen consumed was used in the oxidation of this component. Acetoacetate could not be used by this gland. During ketonuria in the cow, diacetic acid was the predominant ketone body in the urine although the rise in blood acetone bodies was entirely due to a rise in betahydroxybutyric acid (116). When ketosis occurred, it was traced to low carbohydrate intake; it could be dramatically relieved by pumping glucose into the rumen.

Several reviews on various topics in carbohydrate metabolism have appeared during the year (117 to 120).

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FAT METABOLISM[1]

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In spite of the unavailability of most foreign journals and the turning of many American workers to defense activities, many excellent papers on the subject of fat metabolism have been seen during the past year. It has not been possible to refer here to every paper published. Only those have been cited which seemed directly concerned with the topic under discussion.

Absorption

The very interesting postulate of Frazer and co-workers that unhydrolyzed fat can be absorbed from the intestine has been given additional support (1). These investigators report that if liquid paraffin is emulsified (average droplet diameter, 0.5 μ) with a small amount of oleic acid and cholesterol in an alkaline medium and then placed either in the stomach or small intestine, it is readily absorbed. An observation that is not consistent with Frazer's views, but one that might be explained by species difference, was reported by Reiser (2) who found that three to five hours after the ingestion of a fat meal by swine the increase in duodenal mucosal lipids was due almost entirely to free fatty acids. Contrary to this, it has been observed in rats (3) that only 8 to 15 per cent of the acetone-soluble lipids of the intestinal mucosa are free fatty acids. This value does not show any consistent change during fat (Mazola) absorption.

The role of the bile acids in fat absorption still remains obscure. According to Frazer's (1) concept there is no need to postulate a fatty acid-bile salt complex. Furthermore, Virtue *et al.* (4) have found that sodium oleate is readily absorbed from jejunal loops in dogs, and while gall bladder bile enhances absorption, the sodium salts of taurocholic and glycocholic acids are without effect. Weil & Russell (5) believe the changes they observe in plasma phosphatase and lipids are due to the cessation of absorption of certain essential fats

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by rats with ligated bile ducts. With the addition of 3 per cent of ox bile to the diet (Purina dog chow), the phosphatase returned to normal but the blood lipids remained high.

Largely because of early experiments of Munk on a patient with a chylorous fistula it has been tacitly assumed that the majority (60 per cent) of absorbed fat passes into the blood stream via the thoracic lymph duct. Little & Robinson (6) reinvestigated the lymphatic transport of absorbed lipids by collecting left thoracic lymph in dogs after injecting cream into the duodenum. They found that only 4 to 17 per cent of the absorbed fat was recoverable in the thoracic lymph, and they suggested that fat entering the general circulation during absorption might do so by way of lymphaticovenous communications. An interesting observation on the hemolytic properties of lymph (shown to be due to free fatty acids present during fat absorption) was made by Freeman *et al.* (7), who found that in bile fistula dogs the administration of a high-fat diet increased the daily bile pigment output and from this concluded that red cell destruction probably proceeds at a higher rate than on a low-fat diet.

A lower rate of fat absorption by adrenalectomized animals has again been recorded by Bavetta & Deuel (8). The control rats were of a different size from the operated ones and the results are calculated to unit surface area on the assumption that the rate of fat absorption is proportional to body area. Barnes, Rusoff & Burr (9) have repeated their earlier experiments and have again found that with rats of equal size there is no demonstrable difference in the rate of fat absorption by normal and salt-maintained adrenalectomized animals. Another difference in the experiments of these two groups of investigators is that in most cases smaller (younger) rats, which are known to be more susceptible to the harmful effects of adrenalectomy, were used by Bavetta & Deuel than were used by Barnes and his coworkers.

Digestibility and Excretion

A few digestibility studies may be cited as being of especial interest in this period of new wartime foods. Hoagland & Snider (10) have continued their studies with rats and compared the digestive coefficients of lards with hydrogenated vegetable shortenings and shortenings made of animal and vegetable fats. This work is of especial value because complete fatty acid analyses and physical constants of the fats are given. The digestibilities are corrected for endogenous fat

(fecal fat of rats on fat-free diet). The average values given are: lards 94 per cent; vegetable shortenings 87.5 per cent; and vegetable and animal shortenings 85.6 per cent. They find no correlation between melting point or fatty acid composition and digestibility. The melting points lie between 39° and 56° C. Hence these relatively large differences between a natural fat and "synthetic" shortenings are striking and need further elucidation. In studies with men, Sealock, Basinaki & Murlin (11) found 95.7 to 97.4 per cent digestibility of fats in mixed diets containing whole wheat and white breads. The higher indigestible residue of the whole wheat products did not interfere with the absorption of either carbohydrate or fat. It was also found by Hamilton (12) that the addition of 20 to 30 per cent of glucose to a mixed feed did not change the digestibility by sheep of the ether-extractable fat (average 74.3 per cent).

Since the work of Schoenheimer, it has been generally accepted that of all the sterols only cholesterol is absorbed. Now Rosenheim & Webster (13, 14) postulate a new dietary factor in brain tissue concerned with the formation of coprosterol from cholesterol. When dogs were fed a synthetic diet containing cholesterol in lard, 85 per cent of the cholesterol was excreted unchanged, but if Thudichum's white matter containing cerebrosides was fed, the cholesterol was almost quantitatively changed to coprosterol. They do not think this was due to an effect on the intestinal flora. A similar effect was found with β-sitosterol. If this change requires absorption and reexcretion then the plant sterol must be largely absorbed.

Fats have been successfully administered by intravenous injection in a mixed food with casein and glucose (15). As much as 70 gm. of fat were thus given in a day and the weight of the patient maintained.

Relative Nutritive Value of Fats

Further evidence has been given that diets containing appreciable amounts of fat are superior to those comprised almost entirely of carbohydrate and protein. Maynard & Rasmussen (16) show with mixed diets of natural foods that the one containing the higher percentage of fat gives superior growth of nursing young. A second experiment with purified diets may have been affected by an inadequacy of linoleic acid. The data are shown to be statistically significant although it is known that good lactation performance is possible on a diet containing no fat except 50 to 100 mg. daily of linoleic acid (17). Sure (18) found diets containing 15 per cent of lard, butter fat,

Crisco, olive oil, or wheat germ oil equally effective in promoting lactation. Hoagland & Snider (10) compared diets containing 5 or 15 per cent of various lards or manufactured shortenings. The average growth rates of rats for all of the lards were essentially the same as the average for all of the shortenings. However, an examination of their data shows relatively large differences among groups. For instance, on lard 4027 the rats weighed 280 gm. and on lard 4028 they weighed only 245 gm. When adjusted to equal food consumption the differences were no longer statistically significant. However, appetite reflects an important quality of diet. Forced feeding experiments have made it clear that deficiencies which lower food intake (appetite) can be overcome. Hence the material differences between diets fed *ad libitum* must be considered of prime importance. No known physical or chemical property of the individual fat was found to be correlated with its dietary value.

Probably it is this sort of unknown quality in fats that has led to much conflicting data from different laboratories. Again this year several comparisons have been made between butter and other fats without giving a clear decision as to the cause of the differences. Boer (19) postulates a new growth factor in butter acids because his rats grew more rapidly on these than on olive oil. Opposed to this is the finding of Euler, Euler & Säberg (20) that margarine promotes better growth than butter fat. The latter experiment was done by homogenizing the fats into skim milk and is, therefore, more readily comparable to the work of Boutwell *et al.* (21) who found appreciably less growth on corn oil than on butter fat homogenized into skim milk. Several fats were compared in this way and no consistent difference was found due to fat source or unsaturation. Soybean and cottonseed oil are superior to corn oil. However, a special value is attributed to the higher saturated butter acids—a conclusion based on the improvement by hydrogenation of the unsaturated fraction. The most striking results from this kind of experiment have been obtained by feeding calves various fats homogenized into skim milk (22). With this experimental animal there is a definite deleterious effect of all highly unsaturated oils, which uniformly cause decline and death.

Commercially produced monoglycerides of stearic and linoleic acids have been found to produce good growth if fed to rats at low levels (23). However, they were not well tolerated when they constituted 24 per cent of the diets, a level at which lard and other good fats produced optimum growth.

The Essential Fatty Acids

Smedley-MacLean, Hume, and Nunn (24, 25, 26), in continuing their work on the function of the essential fatty acids, have brought out some more interesting material. It is first shown that when rats were kept on a fat-deficient diet for four months their body fat was normal in amount but very low in arachidonic acid (polybromides). This was especially true of the subcutaneous fat which was almost totally devoid of this acid. Then when a minimum dose (one fourth drop daily) of arachidonic acid was fed none of it appeared in the adipose tissue but was held by the liver. They conclude that arachidonic acid is necessary for new tissue and that the subcutaneous tissue is depleted in furnishing a supply to the rapidly growing parts (24). To test this they implanted Walker tumors in young rats on a fat-rich diet (25). The tumors were well developed in ten to fourteen days; then the rats were killed and analyzed. As compared with controls killed on the day of tumor implantation they found no real differences in total fats or the highly unsaturated acids except in subcutaneous tissue. The tumors themselves had a content of arachidonic acid similar to that of the liver while the subcutaneous fat was somewhat depleted. They then implanted tumors in young growing rats on a fat-deficient diet (26). Tumors developed as well as in a group receiving linseed oil so it was not possible by this means to limit tumor growth. On analysis it was found that tumor development had greatly depleted skin and carcass of total lipid and that the subcutaneous fat had lost much of its arachidonic acid, which must have gone into the tumors.

On analyzing the livers of the rats receiving linseed oil they found a preponderance of ether-insoluble bromides which corresponded to clupanodonic acid rather than arachidonic. The source of arachidonic acid in tissues is not known but we now have confirmation that structurally arachidonic acid is 5,8,11,14-eicosatetrenoic acid (27).

An interesting point is brought out by Hilditch & Pedelty (28) who observe that with increasing age the octadecadienoic acid of sheep body fat decreases while the highly unsaturated C_{20-22} acids increase slightly. Sheep fats resemble other animal fats in their content of an octadecadienoic acid which differs from the ordinary linoleic acid of seeds.

Quackenbush, Steenbock and co-workers (29) have also analyzed the tissues of rats with varying degrees of scaly skin and acrodynia,

wholly or partially cured by B-complex or linoleic acid. This was done in an attempt to explain the interrelation between linoleic acid, pyridoxine, and pantothenic acid in their effects on the skin. Although there were some differences in fat content and iodine number of fats in the different groups, there was no evidence that pyridoxine or Vitab brought about a synthesis of linoleic acid in fat-deficient rats. In further study of these interrelations the authors (30) now conclude that none of the B-complex will replace linoleic acid. Their results confirm earlier findings by other workers that in the presence of adequate linoleic acid rats will die of pyridoxine deficiency without developing acrodynia. Pyridoxine, pantothenic acid, and linoleic acid all supplement each other when fed at suboptimal levels but only linoleic acid can alone cure all skin symptoms. With the diets used they found a slight scale of the hind feet which led the authors to suggest that another factor was lacking which had some effect on the skin. While working with pyridoxine Daniel, Kline & Tolle (31) discovered a convulsive syndrome in deficient nursing young. Apparently linoleic acid has no more effect on this syndrome than it does on the decline and death of adult animals. The linoleic acid seems highly specific for maintenance of healthy skin, but it can in no way diminish the need of pantothenic acid, pyridoxine, and other vitamins requisite for certain life processes.

Quackenbush, Kummerow & Steenbock (17) conclude from an extensive study that for normal reproduction and lactation linoleic and arachidonic acids are equally effective as supplements to a fat-deficient diet. Linolenic acid was very inferior for curing their deficient rats as measured by growth, skin clearance, or reproduction. Although they fed the curative acids at only one level (100 mg. daily), the lactation performance of their rats was so superior to that in earlier studies by other workers in which only 25 to 50 mg. of linoleic acid were used that they suggest 100 mg. daily as the minimum optimal intake. This amounts to about 1 per cent of the diet of the rat and is probably in excess for early growth of rats. This is brought out anew by the experiments of Hoagland & Snider (10) who find no evidence that a diet containing 5 per cent of a fat carrying 10 per cent linoleic acid is limited by that fatty acid. However, it should be clearly understood that early growth of a well-fed weanling rat is not greatly retarded on a fat-free diet because of the large storage of linoleic acid.

The skin of fat-deficient rats has been described with camera lucida drawings by Williamson (32). The deficient rats have thicker and

more differentiated epidermis with the stratum granulosum becoming especially distinct and the horny layer thick.

Unsaturated fatty acid deficiency has now been described in mice (33), which develop scaly skin, decline in weight, and respond to a few drops of fat in the same way that rats do. Mice differ from rats in that with combined pyridoxine and linoleic acid deficiency they decline and die before developing either scaly skin or acrodynia (34).

In searching for specific functions of the unsaturated acids, Engel (35) has found that linoleic acid is necessary for completely clearing fatty livers. With synthetic vitamin supplements and a fat-free diet choline had its optimum lipotropic effect only when inositol, pyridoxine, and linoleic acid were all three present. One-tenth milliliter of corn oil daily reduced the liver fat to half, in one experiment. The author postulates a mutual influence of these factors in mobilization of fat. Weil & Russell (5), continuing their earlier studies, find a marked drop in blood phosphatase in rats with ligated bile ducts. They interpret their results as meaning that essential unsaturated fats are not absorbed in the absence of bile but no information is given as to the nature of the fatty acids. Barnes, Rusoff & Burr (36) find that linoleic acid deficiency does not materially decrease the rate of incorporation of labeled fatty acids into liver phospholipids.

The Effect of Dietary Fat on Other Nutrients

Additional information concerning the mechanism of the well-known antirachitic effect of fat has been given by Jones (37), French (38), and Booth, Henry & Kon (39). Independently the first two investigators have established the fact that dietary fat increases the acidity of the intestine, in particular the jejunum and lower ileum. Although this change in acidity cannot be the explanation for the antirachitic action of vitamin D and of phosphate, it does explain the increased absorption of calcium and phosphorus during fat feeding. The ingestion of fat has little if any effect upon the pH of the upper duodenum (37, 40). Booth and co-workers (39) have found that on a high calcium—low phosphorus diet calcification of bones is definitely aided by the presence of fat in the diet. The effect is not evident with low calcium—high phosphorus diets. Dietary fat lowers the excretion of phosphorus in the feces and of calcium in the urine. They call fat a phosphorus saver but they express the view that the true mechanism of the fat effect is still obscure.

Many conflicting reports still appear in the literature concerning

the effectiveness of vitamin A when administered in different fats. These differences may be accounted for by the observation that fat is necessary for the optimal absorption of vitamin A (41) and carotene, and that there is a rapid destruction of both in the intestinal tract. This destruction can be largely inhibited by the simultaneous administration of α-tocopherol or other fat-soluble antioxidants that normally occur in certain fats (42, 43, 44, 45). Water-soluble polyphenols are very effective in protecting carotene in oils *in vitro,* but due to their ready extraction by water from oil emulsions oxidation of carotene is not greatly inhibited by them in the intestinal tract. Since vitamin A is largely absorbed within a few hours (46), its loss in the intestine is so rapid as to suggest the action of an enzyme of the fat oxidase type (see p. 175).

The absorption of crystalline carotene by hens is much more effective on a normal than on a low-fat ration (47) : vitamin A dissolved in a trace of fat was absorbed equally on the two rations. The fall in blood and butter carotene in cows following the ingestion of shark liver oil seems to be due to the vitamin A rather than the fat *per se* (48). No toxicity comparable to that of cod liver oil was observed.

Mattill & Golumbic (49) again emphasize the point that no distinction need be made between the cod liver oil–induced muscular dystrophy and that produced by simple vitamin E–free diets. Even with antioxidants in the oil, mixed rabbit diets retain their vitamin E only temporarily. Eppstein & Morgulis (50) have demonstrated that the daily feeding of cod liver oil separately (1 ml. per kg. body weight) quickly induced dystrophy in rabbits. Hence the cod liver oil must have brought about destruction of the vitamin in the intestine or enhanced dystrophic symptoms in some other manner.

In feeding simplified diets containing refined fats which are not stabilized care must be taken to prevent rancidity effects which will lead to erroneous conclusions. Frequent mixing of diets, refrigeration, and if necessary the addition of antioxidants is to be recommended. Although it has been reported that hydroquinone in the diets of cats or mice brings about graying of the hair, this effect could not be repeated by Henderson *et al.* (51) who found no ill effects of daily doses of 25 to 35 mg. Overman (52) fed 1 mg. daily for a period of two months without finding any effect on the stability of the rat body fats. She found that ascorbic acid in the diet appreciably stabilized the body fats but the results were not entirely regular.

Fats which have been heated in a thin layer until they have very

high peroxide values are in themselves quite toxic when injected intraperitoneally (45). The presence of 1 to 5 per cent of thymol during heating markedly reduces the toxicity as well as the destructive action on vitamin A, which affords further support to the view that added antioxidants are to be preferred to rancidity. These authors conclude, from indirect measurements, that the inclusion of soybeans in the diet of the hog does not decrease the stability of the lard.

The thiamin-sparing action of protein is reported by Wainio (53) to be approximately proportional to its nonglycogenic fraction. However, the work of Banerji & Yudkin (54) would indicate that protein may be as effective as fat in this regard. In the latter work the diet was entirely free from carbohydrate. Neither high-fat nor high-protein diets eliminate the "catatorulin effect" although the outward symptoms of thiamin deficiency are completely prevented.

Levels of dietary fat have been shown not to affect the riboflavin requirement of the dog (55)—a result in contrast to the increased need by rats on a high-fat diet. Dietary fat has a slight sparing action on the pantothenic acid (56) and biotin (57) needs of the rat.

A very striking effect of large amounts of dihydroxystearic acid in the diet has been shown by Lockhart, Sherman & Harris (58). The test rats did not grow well and developed hemorrhages which were prevented or cured by vitamin K. Apparently this unnatural fatty acid induced the deficiency in rats which would have remained normal on the basal diet low in vitamin K.

Synthesis, Deposition, and Mobilization

Adipose tissue.—Hilditch & Pedelty (28) have studied the composition of perinephric and external tissue fats of sheep undergoing rapid gains or losses in weight. Contrary to earlier observations made on other animals no definite differences in deposition or mobilization rates of the individual fatty acids were observed. Braun & Shrewsbury (23) have fed low levels of commercial monoglycerides of stearic and impure linoleic acids. The rats did not become as fat as their controls on lard. No monoglycerides were found in the body tissues and stearic acid, unlike linoleic, failed to be deposited.

MacKay, Barnes & Carne (59) have shown that the replacement of dietary carbohydrate with protein results in a decreased appetite, smaller weight gain, and less body fat. Additional evidence has been presented by Pauls & Drury (60) that the large quantity of sugar stored in the diabetic animal when given insulin is changed to fat.

This observation is used to support their hypothesis that one of the major functions of insulin is to effect the conversion of carbohydrate to fat.

Indirect support of the hypothesis that there is an increase in C_{16} fatty acids when fat is being formed from carbohydrate has been given by Samuels, Reinecke & Ball (61), who found that the mean molecular weight of rat body fat was lowered by a high carbohydrate diet.

These same authors (62, 63) fed by stomach tube equicaloric amounts of high-fat and high-carbohydrate diets to hypophysectomized rats. The carbohydrate-fed hypophysectomized rats stored more fat in the peripheral tissues than did normal controls while the fat-fed rats showed no such difference. The greater ability of the fat-fed rats to maintain blood sugar levels was accompanied by greater liver glycogen supplies and increased insulin tolerance after starvation (64). The interpretation given these studies is that the metabolic disturbances of the hypophysectomized rat are due to an increased ability to convert carbohydrate to fat or a decreased ability to mobilize stored fat.

A possible role of the androgens in the mobilization of depot fat is suggested by the study of Turner & Mullikin (65) in which testosterone, injected into castrate male mice, increased the rate of disappearance of subcutaneously injected corn oil.

Several reports have been made concerning experimental hypothalamic obesity in the rat (66, 67, 68), in the dog (69), and in the monkey (70). Some of these observations (67, 68) indicate that the development of obesity is largely due to an increased appetite, but a lowered total metabolism through decreased spontaneous activity seems to be a factor (67). Block (71) found no evidence to support Bergmann's lipophilia hypothesis of obesity in man.

The effect of vitamin deficiencies on fat synthesis has been further studied by Quackenbush, Steenbock & Platz (72) who find that when a diet is made complete, whether by thiamine, pyridoxine, or pantothenic acid, renewed growth is accompanied by a severalfold increase in body fat. The evidence is clearly against the view that thiamine more than the other vitamins is specifically concerned with fat synthesis.

Blood lipids.—In an extensive study of normal blood lipid values Boyd (73) has presented data for seven different lipid fractions from seven animal species. The sphingomyelin of various tissues of the cat ranges from about 7 to 33 per cent of the total phospholipids (74).

There are many conflicting reports in the literature concerning the effect of the nutritional state of an animal upon the level of blood lipids. Entenman & Chaikoff (75), in a series of investigations on this subject, have found that overfeeding of dogs, resulting in varying degrees of obesity, did not affect in any regular manner the level of blood lipids. Furthermore, upon fasting there was no rise in blood lipids of either normal or obese dogs. In fact, there was usually some decrease—an observation not in accord with the findings of Block (71) with human subjects. The blood lipids of calves on a low-fat diet fall to surprisingly low levels (22). In the absence of bile the marked rise in rat blood lipids is attributed to failed absorption followed by mobilization of the body stores (5). The feeding of bile does not correct this condition.

Rossen & Reichenberg (76) found a slight tendency for blood total fatty acids to rise following the administration of large doses of vitamin E to male schizophrenics. Dam & Kelman (77) observed an increase of 20 to 40 per cent in the ratio of phospholipids to total blood lipids of fasting chicks when the diet contained vitamin E or lipocaic. Addition of cholesterol to the diet depressed the phospholipid ratio. In dogs given a pantothenic acid–deficient diet blood lipids fell as much as 50 per cent and livers became extremely fatty (78). The feeding of lecithin to rabbits receiving cholesterol lowered the blood cholesterol and decreased the incidence of atherosclerosis (79).

In studies on the lipemia of nephritis it has been found that if dogs are bilaterally nephrectomized or poisoned with mercuric chloride, potassium dichromate, or uranium nitrate, there is a marked rise in blood lipids (80). Also, rats with severe nephrotoxic nephritis, induced by antikidney serum, develop a lipemia (81). The close correlation between hepatic damage and the change in ratio of free to total blood cholesterol has again been pointed out (82).

Flock & Bollman (83) have made a detailed study of the hyperlipemia that is exhibited by cocks when given diethylstilbestrol. The great rise in blood neutral fat that occurs is interpreted to be brought about through mobilization from the depots, but the increase in phospholipids is probably too great to be accounted for solely on this basis. It is proposed that the increase in blood phospholipids represents a new synthesis. Even though there are newly synthesized phospholipids that accumulate in the blood it is not possible to tell whether the increase has been brought about by a stimulation of synthesis or an inhibition of phospholipid destruction through estrogen action. Loeb

(84) has found a moderate lipemia in another species, the rat, following the injection of an estrogen (estradiol benzoate).

In studies on the effect of the pancreas on blood lipids, Gibbs & Chaikoff (85, 86) have found that depancreatized dogs receiving insulin but no raw pancreas in their diet have low blood lipid values. When insulin was withheld there was no regular occurrence of hyperlipemia. If these dogs were fasted while insulin was being withheld there again was no change in blood lipids. However, if the diabetic dogs were given raw pancreas there was a hyperlipemia before and during insulin deprivation. If these pancreas-fed depancreatized dogs were deprived of insulin and then fasted there was a fall in blood lipids.

The role of the thyroid in regulating blood lipids has been investigated by Entenman, Chaikoff & Reichert (87, 88). It was found by them that the rise in blood lipids in the thyroidectomized dog can be prevented by restriction of the caloric intake or by fasting (87). However, it is interesting to note that the great fall in fatty acids and cholesterol esters which represents the largest alteration during fasting in the thyroidectomized dog brings the blood lipid level down to the range of the normal fed animal but does not quite reach the level of the normal fasted dog. If the hypophysis is removed from the thyroidectomized dog and a constant caloric intake is maintained, there is found an elevation in blood lipids which is thought to be due to the lack of thyroid and is not influenced by hypophysectomy (88). The injection of rabbits with thyrotropic hormone of the anterior pituitary may reduce the blood cholesterol about 25 per cent (89). Testosterone propionate and estradiol dipropionate lessened the hypercholesterolemia and aortic changes in male rabbits fed cholesterol (90). The effect was not seen with females.

Liver fat.—A number of reports have been made concerning the dietary cirrhosis and hemorrhagic necrosis that develop in the livers of animals fed on various regimens. A low-protein, low-choline diet which is known to cause fatty livers also brought about the development of hepatic cirrhosis when fed over a sufficiently long period of time (100 to 150 days) (91 to 96). Rabbits fed an otherwise adequate diet developed cirrhosis if 20 per cent butterfat or cottonseed oil was included (97). Cystine additions to the diet aggravated the cirrhosis of the low-protein, high-fat diet and produced a cirrhosis in rats when fed an adequate stock diet (98). These effects have been used to distinguish cirrhosis from hemorrhage and necrosis (93, 96). The increased choline requirement with added cystine is attributed to a

better nutritional state rather than an antagonism (99). There is no correlation between liver fat and the degree or incidence of cirrhosis (97, 98), but it is apparently true that those dietary conditions which cause the development of cirrhosis will also bring about fat infiltration even though the fatty changes might not be present at the time cirrhosis develops. Furthermore, those agents which prevent cirrhosis, i.e., choline, methionine, protein, and yeast, are also effective in preventing fatty livers.

The influence of liquid unsaturated, solid unsaturated, and solid saturated fatty acids incorporated in a high-fat, low-protein, low-choline diet on the extent of fat infiltration into the liver was studied by Channon, Hanson & Loizides (100). Additional evidence has been reported that brominated fatty acids in the diet cause the development of fatty livers and that bromine substituted in the carbon chain of the following fatty acid esters produces fatty livers in the order of decreasing activity: 9,10-dibromostearic, 13,14-dibromodocosanoic, 6,7-dibromostearic. 10,11-Dibromohendecanoic ester was found to be highly toxic (101).

Starvation studies with mice have indicated the following changes in liver lipids (102). During the first two days of fasting an excess of neutral fat was deposited in the liver. Accompanying this fat increase there was a decrease in the iodine number. The weight of phospholipid decreased, but as the total liver weight also decreased there was no change in the percentage concentration.

Active interest in the field of lipotropic substances and the mechanism of lipotropic action has resulted in many new advances. However, in this section only those studies which deal directly with fat transport will be discussed.

The earlier postulate that for methionine to exert its full lipotropic action some other protein constituent must be present has not been confirmed (103). On the contrary it was found that diets which were supplemented with the free amino acid were superior in lipotropic action to diets containing equivalent amounts of methionine in casein.

In rats on a choline-deficient diet it has been reported that there was a decrease in the concentration (but not in absolute amount) of total phospholipids in the liver and kidney (104) and a decrease in the concentration of choline in the fresh liver (105, 106). However, Jacobi & Baumann (107) report that the choline content of "choline deficient" rats is slightly higher than normal. Stetten & Grail (106)

found that only the markedly fatty livers resulting from feeding guanidoacetic acid were very poor in choline, presumably because of irreversible transmethylation to form creatine. Another explanation offered for the retention of choline by fatty livers is the inhibition of choline oxidase in these tissues (108).

The lipotropic action of inositol has been substantiated (109, 110, 35). In a comparison of the relative effectiveness of inositol and lipocaic it was concluded that although inositol was in some respects similar to lipocaic, this could not be the entire explanation of lipocaic action (110). Animals deficient in the essential fatty acids (35), pantothenic acid (78, 111), or riboflavin (55) develop fatty livers.

Popper & Necheles (112) have further studied the controversial question of the role of the pancreas in the fatty liver syndrome. They found that if all pancreatic ducts were ligated, the pancreas atrophied and very fatty livers developed within twelve months. On the other hand, if only the main ducts were ligated, the pancreas again atrophied, but no fatty change in the liver was noticed. Their conclusions from these experiments are that the fatty livers were due to a lack of external pancreatic secretion, and that only a small pancreas and probably a small secretion is necessary to prevent the liver changes.

The involvement of the sex hormones in the production of fatty livers has been indicated by MacBryde *et al.* (113), who report that estrogenically equivalent doses of estrone, estradiol benzoate, and diethylstilbestrol resulted in fatty and hydropic degeneration in the liver of the dog.

Miscellaneous.—The synthesis of the bases found in phospholipids has been studied by Stetten (114, 115) who used ethanolamine containing N^{15}. Serine with heavy nitrogen was obtained after three days of feeding ethanolamine, and isotopic choline was abundant in rats so depleted of methyl as to have fatty livers. Several compounds will prevent fatty livers without being active methyl-donors in the synthesis of methionine—the more specific reaction (116). Arsenocholine enters directly into the synthesis of lecithin and prevents fatty livers but is not a methyl-donor (117). This suggests that the lecithin molecule, rather than available methyl, is essential to lipotropic action.

There is recent confirmation of the observation that the rate of phospholipid synthesis by the liver is not altered by adrenalectomy (118), which is known to interfere with transport into the liver. Stewart & Sinclair (119) have also shown that ricinoleic acid is readily absorbed by the rat, wherein it is metabolized and transported

into neutral fat stores without being detectable in phospholipids. The available evidence makes difficult the acceptance of the general thesis that phospholipid synthesis is an essential part of fat transport.

Eggs laid by hens for eight days after intramuscular injection of P^{32} were found to contain lecithin and cephalin with identical amounts of the isotope (120). However, in liver phospholipids analyzed four hours after the administration of radio-sodium phosphate the cephalin was somewhat more active than the lecithin—a difference that disappeared in twelve hours (121). The relative rate of incorporation of P^{32} into sphingomyelin of liver was low; of muscle, high. Very large differences in the rates of transfer of tagged phospholipids across membranes have been observed (122, 123). In a series of studies by Chaikoff and co-workers (124, 125, 126, 127) on the incorporation *in vitro* of P^{32} into phospholipids of brain and liver tissues, it has been established that the intact, normally respiring cell is most active. Homogenization, lack of oxygen, lack of glucose, and the presence of respiratory poisons wholly or partially inhibit the reaction.

No gain or loss of cholesterol takes place during sterile incubation of rat liver or brain tissue (128). Block & Rittenberg (129) have found that rats and mice produce deutero-cholesterol from fed deuteroacetate but not from propionate, butyrate, or succinate. The absence of deuterium from the body fatty acids makes unlikely their use for cholesterol synthesis. In these experiments at least 13 per cent of the hydrogen atoms of cholesterol came from the acetate.

Utilization and Intermediary Metabolism

In a continuation of his balance studies on carbon-tetrachloride–poisoned rats, Winter (130) has confirmed previously made observations that there is a decrease in fat utilization associated with this type of liver injury.

An enzyme and activator have been isolated from beef liver which catalyze the dehydrogenation of lecithin with methylene blue as a hydrogen acceptor (131). As dehydrogenation was accompanied by an increase of iodine number it is probable that the unsaturation was not in the α-β position. Additional evidence that in biological oxidation a cleavage of double bonds located in the center of the fatty acid carbon chain is not a normal pathway has been presented by Bernhard (132). Using deuterium as a tracer he found that adipic, suberic, sebacic, and higher dicarboxylic acids were not metabolized to any

appreciable extent. These experiments not only throw doubt on the central cleavage of fatty acids, but also strengthen the view that ω-oxidation cannot be a predominant process in the catabolism of fat.

As evidenced by the relatively large number of reported investigations, acetone body metabolism still remains of major interest. It appears that an inclusive reinvestigation of possible acetone body precursors among the amino acids is being undertaken by several investigators. Tryptophane (133) and lysine (134) were studied and found to be without effect on the urinary excretion of acetone bodies. Although the excretion of acetone bodies has been the most widely used method of identifying possible precursors, it should be kept in mind that acetone body production by the liver might be increased but not reflected in the urine output. This has been well demonstrated in the recent observation that the odd-carbon fatty acids are acetone body precursors even though their administration to an animal does not increase acetonuria.

In the past few years there has been an accumulation of evidence supporting the modified β-oxidation theory. According to this scheme, the two-carbon compounds formed by β-oxidation condense to acetone bodies. Although it is not known what two-carbon compound is formed, final proof that one such compound, namely, acetic acid, can be converted into the acetone bodies has been established by Swendseid *et al.* (135). In the same study, which employed heavy carbon as a biological tracer, it was found that sodium bicarbonate, which causes an increase in acetone body excretion, does not itself contribute to the synthesis of these compounds. MacKay and his collaborators (136, 137) have found that neither the amount of liver fat nor substances which affect it have any significant influence upon the degree of fasting ketosis in the rat. Furthermore, the type of fat in the diet preceding fasting is of no significance. Protein is the important dietary constituent in suppressing the ketosis of a subsequent fast—an effect thought to be mediated through "stored" protein which helps to maintain liver glycogen levels during periods of starvation. The protein effect has also been demonstrated by Shipley (138) in both normal and hypophysectomized rats.

An interesting effect of glucose on acetone body metabolism has been shown by Somogyi & Weichselbaum (139). Data are presented by these authors on blood acetone bodies of diabetic humans and depancreatized dogs in varying states of ketosis at short intervals (up to six hours) after the ingestion of a given dose of glucose. In some

cases a rise in blood acetone bodies followed glucose ingestion, and this was interpreted to indicate that in these individuals liver glycogen was not immediately stored, but that glucose utilization by extrahepatic tissues was increased to the point of sparing acetone body utilization. As the evidence of an increase in blood acetone bodies is not too definite and there is no supporting evidence in the literature for such a sparing effect of glucose on acetone body utilization by muscle tissue, a more definite interpretation of these experiments should await reexamination.

During the year, support has been given to three different theories of the metabolic stimulus for increased acetone body production by the liver. As space does not permit any detailed discussion, their general nature only will be mentioned: (*a*) regardless of the nature of the glycogenolytic factor, an increase in the rate of hepatic glycogenolysis entails an increase in the rate of acetone body production (140); (*b*) when liver glycogen is diminished there is an increase in fatty acid oxidation resulting in an increased acetone body production (141); (*c*) the demand for glucose, or a substitute, by the extraneural tissues that is evident when there is a shortage of carbohydrate for fuel brings into play the supplementary mechanism of acetone body production by the liver (142).

Employing a tissue slice technique, Ennor (143) has observed that the oxygen consumption of fatty livers from guinea pigs poisoned with either carbon tetrachloride or phosphorus is above normal if it is calculated on the basis of fat-free, dry tissue. Equal quantities of acetone bodies were formed from crotonic and butyric acids, the quantity being the same in normal and poisoned livers. Bobbit & Deuel (144) have studied the production of acetone bodies by rat liver slices and the disappearance of butyric acid from the substrate with and without added glycogen. After the experimental incubation period there was no definite difference between the amounts of butyric acid as such that could be recovered from the control and glycogen groups. However, less acetone bodies were found in the glycogen-treated liver slices than in the controls. This was interpreted to indicate that the glycogen brought about a "ketolytic" removal of the acetone bodies, which tacitly assumes the direct and complete conversion of butyric acid to acetone bodies. It should also be mentioned that data from other laboratories show no differences in acetone body production from butyric acid by (*a*) normal and carbon tetrachloride fatty livers (143), (*b*) livers from fed and fasted rats, guinea pigs, or monkeys

(145), and (*c*) livers with and without added glycogen as in the above study (146).

The large proportion of the caloric requirement that can be fulfilled by the utilization of acetone bodies has been again demonstrated (147). In this case utilization by the mammary gland was found to be restricted to the β-hydroxybutyric acid. The nonutilization of acetone bodies by the brain has been confirmed (148), and it has been shown that even in a state of ketosis the dog brain has an R.Q. of approximately 1.0. Friedemann (149) has analyzed data from dogs, monkeys, and humans in varying states of ketosis and has found that the ratio of β-hydroxybutyric acid to acetoacetic acid in the urine increases with the rate of excretion.

The markedly different effects of pancreatectomy in different species has again been demonstrated. The depancreatized monkey exhibits a pronounced ketonemia within two to four days after deprivation of food and insulin although showing only a mild ketonemia when insulin alone is removed (150). On the other hand, the depancreatized duck develops a lower degree of ketonemia when fasted than does the normal duck while phlorhizin administration causes a greater ketonemia in the normal (151). Lipocaic which is known to have no effect on other types of ketosis has been shown to be without influence upon the ketonemia of the diabetic dog (152). The lack of close correlation between the clinical condition of diabetic coma and the level of blood acetone bodies in the human diabetic has been again emphasized (153).

The controversial question of the influence of the adrenal cortex upon ketosis has been reopened by Shipley & Fry (154). They found no stimulation of ketosis by adrenal cortical extract, corticosterone, desoxycorticosterone, or 11-dehydro-17-hydroxycorticosterone (compound E).

Chemical and Enzymic Studies

Although it is not the prerogative of this review to discuss the chemistry of the lipids, a few citations will be made of work which may directly affect the interpretation of metabolic studies. The presence of inositol in phospholipids (155) may explain its lipotropic action. Since on purification phosphatidyl serine and phosphatidyl ethanolamine have properties quite different from the impure cephalin (156), analytical procedures based on solubilities may have to be re-

vised. Sinclair & Dolan (157) have re-examined the conditions for separating the phospholipids and conclude that the so-called ether-insoluble portion is not a special lipid but simply a fraction of the whole. Isolation of a dihydrosphingosine from brain (158) and the demonstration by the method of isotope dilution that ethanolamine, choline, and serine in no case account for 100 per cent of the nitrogen of impure acetone precipitates (159) further emphasize the complexity of the analytical fractions now being studied. Folch (160) has given proof that because of rearrangement of glycerophosphoric acid in the presence of acid and alkali, available data do not provide evidence as to whether the glycerophosphoric acid in phosphatides is the α- or β-form. Isomerization of fatty acids may also take place in strong alkali or on adsorbents. Mowry, Brode & Brown (27, 161) find a small percentage of conjugated material in their preparations of arachidonic acid. Its alkali isomerization takes place in a manner comparable to that of linoleic and linolenic acids. A very small percentage of conjugated material also develops in oils during bleaching with fuller's earth (162), and many reagents isomerize and polymerize fatty acids and carotenoids (163). An attempt to prepare more useful derivatives of the octadecenoic acids failed to give satisfactory results (164). As a possible source of error in tissue lipid analyses Gomori (165) points out that lipid aldehydes (plasmal) react to Bennett's histochemical test used for ketosteroids. The plasmal derivatives isolated by Ancher & Waelsch (166) seem to be largely stearyl and palmityl aldehydes. Edman's micro-method for cerebrosides is based on the determination of galactose (167).

Strain (168) has shown that fat oxidase is specific for the *cis*-configuration of the 9 : 10-ethylenic linkage in fatty acids. Sumner (169) has measured the degree of peroxidation (per cent of total possible) of several fatty acids in the presence of lipoxidase preparation. Whereas oleic acid gave only 16 per cent peroxidation, linoleic and linolenic acids rapidly attained 90 and 95 per cent, respectively, for one double bond. He also shows that carotene is oxidized by active intermediates of peroxidation of fatty acids, not by the peroxides themselves (170).

Bergström & Wintersteiner (171, 172, 173), in continuing their studies of the autoxidation of colloidal cholesterol solutions, have shown that cyanide completely inhibits the reactions. Numerous inhibitors and accelerators were studied. As is the case with fatty acids, the esters are more stable toward oxygen than the free alcohol.

Golumbic (174) has concentrated alkali-labile red antioxygenic

materials from plant oils which are similar to chroman-5,6-quinones but have no vitamin E activity.

Handler & Bernheim (108) show that in fatty livers the choline oxidase is greatly reduced or inhibited and they offer this fact as a possible explanation of the high choline content even in the absence of available methyl. Trypsin is completely inhibited over a two-hour period by soaps of all liquid fatty acids in concentrations of about 4×10^{-5} *M* (175). In conclusion it might well be pointed out that all of the observations on autoxidation, antioxidants, and lipid-enzyme relations must be given special consideration when *in vitro* studies are being conducted.

A number of interesting reviews have appeared during the year which are cited at the end of the bibliography (176–180).

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THE METABOLISM OF PROTEINS AND AMINO ACIDS

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Theories of Protein Metabolism

This year is an appropriate one for reviewing the evidence which invalidated the classical theory of protein metabolism (in animals) epitomized in the terms "endogenous" and "exogenous" metabolism; the evidence which has established the theory proposed by Borsook & Keighley (1) and confirmed and extended by Schoenheimer and his colleagues (2) and by Tarver & Schmidt (3) will also be reviewed in this context.

According to the classical theory of protein metabolism, protein synthesis, in the adult organism in nitrogen equilibrium, is restricted to the replacement of an unceasing "wear and tear" or endogenous quota. This quota corresponds approximately to the minimum excretion of nitrogen on a diet containing little or no nitrogen but otherwise adequate. When more than this minimal quantity of protein nitrogen is ingested, the excess over the endogenous requirement is considered to be quickly catabolized and appears in the urine mainly as urea. This theory was presented first in 1905 by Folin (4), and modified in 1912 by Folin & Denis (5).

The experimental basis for the postulate of an endogenous or "wear and tear" quota was Folin's discovery of the constancy of the urinary creatinine and neutral sulfur regardless of the amount of protein in the diet. The urinary creatinine and neutral sulfur, especially the former, were interpreted, therefore, as measures of the rate of endogenous nitrogen metabolism.

The theory was tested experimentally by McCollum & Hoagland (6) and by Terroine *et al.* (7). They argued that when the rate of endogenous nitrogen metabolism is increased, there should occur a corresponding increase in urinary creatinine and neutral sulfur. It was found that the endogenous nitrogen metabolism and, later by Deuel *et al.*, that the basal metabolism (8) can be increased without changing the excretion of creatinine.

The concept of endogenous metabolism, as formulated by Folin, was felt to be inadequate very early in the development of other aspects of protein metabolism. McCollum (9, 10), Osborne & Mendel

(11), and Mitchell *et al.* (12) questioned whether the "wear and tear" quota really involved the destruction of any intracellular protein. The function of the endogenous metabolism, according to these authors, is to supply compounds such as essential amino acids for specific and indispensable but nonprotein purposes. When these amino acids are supplied from the diet there is no need for breakdown of the protein of the tissues.

In 1935 Borsook & Keighley (1) proposed a theory of protein metabolism which is completely in the opposite direction of previous modifications of Folin's theory. In an animal in nitrogen equilibrium, the breakdown of intracellular protein is continually in progress even when abundant quantities of amino acids are obtained from the diet. This breakdown bears no "wear and tear" connotation; it greatly exceeds Folin's endogenous quota and is directly proportional to the level at which the nitrogen balance has been set by the previous dietary history. As a consequence, in nitrogen balance, a corresponding quantity of amino acids is synthesized into tissue protein and peptides. When protein is ingested, in the course of the next twenty-four hour period, some of its amino acids contribute toward maintaining constant the concentration of free amino acids in the blood and tissues. A large fraction is synthesized into protein and peptides while the remainder is catabolized; this nitrogen appears in the urine. The last moiety might be called the "exogenous" quota, distinguishing it from the urinary nitrogen arising from the catabolism of tissue protein and amino acids which is the "continuing metabolism" nitrogen of Borsook & Keighley.

The new theory was based on two lines of evidence. In experiments in which nitrogen balance was maintained for one day with nonsulfur-containing amino acids or ammonia, the sulfur excretion was considerably in excess of the so-called "endogenous" sulfur excretion. In view of the fact that nitrogen balance was maintained, an increased "endogenous" metabolism could not be invoked to account for a sulfur excretion in excess of the "endogenous" level.

Other experiments indicated that, over one day periods, whether the body nitrogen is spared by a protein or by nonsulfur-containing amino acids or ammonia, the same amounts of sulfur and, by inference, of nitrogen are contributed to the urine by tissue sources.

These contributions from the body could not have come from the pool of free amino acids and must have come from protein and peptides because their concentration in the tissues is remarkably inde-

pendent of the dietary state and remains unchanged even in starvation (12 to 16).

Proponents of the classical theory appear to have been unaware that the phenomenon of labile nitrogen or "reserve protein" (8, 17 to 21), as it has been called, was a fundamental discrepancy in view of the constancy of the nonprotein nitrogen in the tissues. There is an implication in the terms "labile" and "reserve" that this protein is somehow different from the main mass of body protein. The evidence for its existence is seen most clearly in the mathematical treatment of the lag in the attainment of nitrogen balance in passing from one level of nitrogen intake to another. Martin & Robison (17) discovered the relation that the utilization of the stored nitrogen follows the course of a first-order reaction. Only the "labile" nitrogen undergoes this rate of catabolism because this "first-order" degradation stops abruptly long before there is any significant loss of total body protein (8).

Other early evidence of the existence of a storage of "labile" nitrogen was furnished by Chambers & Milhorat (18).

As a result, many attempts have been made to distinguish this "labile" protein from "fixed" organ protein. All these attempts have failed (22, 23, 24).

The answer to this difficulty may be that the "labile" nitrogen is not distinguished primarily by a difference in composition but by its location. Some organs, the liver, intestine, and kidney, change in size and protein content quickly with changes in level of dietary protein. The "labile" protein will then arise in the course of a fast, at first from the substance of the more labile organs. But these organs can increase or decrease only within certain limits. Then, as the fast is prolonged and the lower limits of size of the more labile organs are approached, larger amounts of nitrogen will come from the organs slower to change, such as the muscles. The liberated muscle nitrogen will, in part, be resynthesized into liver and kidney protein; the remainder will participate in maintaining the characteristic and constant concentration of free amino acids in the blood and tissues and, in so doing, eventually be catabolized. Thus in the course of a fast, the source of the so-called "reserve" protein will shift from organs which change rapidly to those which change slowly, and hence its composition and the N/S ratio in the urine will change. This explanation is in accord with the failure to identify any definitive "reserve" protein with the changing N/S ratio of stored nitrogen and urinary nitrogen in the course of a fast (8, 25, 26), with the variations in protein

content of different organs in the course of a fast and on subsequent feeding (21, 17, 28), and with the different rates of interchange of isotopes in different organs (2, 3). An explanation similar to the above was suggested independently by Schoenheimer & Ratner (29).

Even before isotopes became available for tracer studies, the existing evidence made it necessary to envisage extensive synthetic processes continually in operation and greatly in excess of those required to replace the losses of the "wear and tear" quota of the classical theory. The new view was considered favorably by Madden & Whipple (30) who concluded from their experiments on the replenishment of plasma proteins lost by plasmapheresis that a dynamic equilibrium exists in the body whereby proteins of the plasma, liver, and other tissues are constantly exchanging.

It was one of the important contributions of Schoenheimer and his collaborators (2) that, employing N^{15} as a tracer, they furnished direct evidence that nearly all the proteins of the body are continually undergoing synthesis and breakdown. In the liver and intestinal mucosa, more than half their protein is broken down and resynthesized in ten days; in the muscles it is slower, and still slower in erythrocytes.

Schoenheimer *et al.* (31), using glycine, *l*-leucine, *d*-leucine, and *dl*-tyrosine labelled with N^{15}, found that the rate of incorporation of dietary isotopic nitrogen in plasma proteins was approximately the same as that in the proteins of the kidney, liver, and intestinal tract. All fractions of the plasma—fibrinogen, euglobulin, pseudoglobulin, and albumin—interchanged N^{15} at about the same rate. In contrast to the plasma proteins, porphyrin and the proteins of erythrocytes exchanged N^{15} very slowly, indicating that the cycle of degradation and synthesis of hemoglobin is much slower than in organ and muscle proteins.

The rate of replacement of nitrogen in antibody protein by dietary N^{15} is the same as in other proteins of the serum. The half-life of antibody and other serum proteins *in vivo* is about two weeks. Antibody proteins, induced by active immunity, are continually being synthesized with the incorporation of dietary nitrogen and continually being broken down at a rapid rate (32).

On the other hand, injected antibody protein (passive immunization) does not incorporate dietary nitrogen [Heidelberger *et al.* (33)]. The N^{15} in passively introduced antibody protein disappeared from the serum faster than N^{15} in the protein of actively induced antibody. The authors concluded that in the absence of an active immune mecha-

nism, antibody protein circulating in the body is catabolized but does not participate in the regenerative uptake of nitrogen.

Tarver & Schmidt (3) found, with radioactive sulfur as a tracer, no interchange of components between the different proteins of the plasma except possibly to a slight extent between the euglobulin and pseudoglobulin. In accord with the findings of Schoenheimer *et al.* (31) they found no uptake of radioactive sulfur by the erythrocyte proteins, i.e., the synthesis of new hemoglobin was too slight in a period of a few days to be detected.

Clark *et al.* (34) observed, in dogs with depleted protein stores and lowered plasma proteins, regeneration of plasma protein by an intravenously introduced enzymatic digest of casein as the sole source of nitrogen.

Tarver & Schmidt showed that no separation can be made between fixed and "reserve," i.e., more labile, tissue protein. They fed methionine-containing radioactive sulfur to rats fasted three to eight days and to dogs fasted eight days and found the radioactive sulfur in the proteins of many organs.

The last remaining experimental support of the classical theory was the fact that the quantity of urinary creatinine is independent of the amount of protein in the diet; it remains undiminished even on a nonprotein diet (8). Bloch, Schoenheimer & Rittenberg (35) found that the urinary creatinine is derived only from the creatine in the tissues and that creatine once formed is not degraded further. The creatinine excreted in the urine in twenty-four hours corresponds to about 2 per cent of the creatine in the body.

The reviewers have found (36) that phosphocreatine, in the range of physiological hydrogen ion concentrations, yields at 38° spontaneously, i.e., without the intervention of any enzyme, free creatinine at precisely the 2 per cent observed by Bloch *et al.* in the body. This is a much faster rate than the formation of creatinine from free creatine under the same conditions. The rate of creatinine formation from phosphocreatine is also identical with the increase in creatinine observed in extracts of muscle and brain by Hammett (37).

The phosphocreatine content of muscle varies little; it then follows, since it is the immediate precursor of the urinary creatinine, that the latter will vary little; the corollary is the creatinine coefficient.

The amount of urinary creatinine in no way characterizes any active process in the tissues. It is a measure only of the level at which

the body maintains its phosphocreatine, i.e., of those synthetic processes over a long preceding period in the course of which phosphocreatine was formed from free creatine and phosphoric acid. It is immaterial to the synthesis of creatine whether the arginine, glycine, and methionine which are its precursors (38 to 43) come from dietary or tissue protein.

The concentrations of protein, free amino acids, and ammonia in the tissues are such that the "steady state" they represent is far from the thermodynamic equilibrium state on the side of protein (1, 44). This "steady state" is a dynamic state maintained by a constant input of chemical potential energy through coupling with oxidative reactions.

The thermodynamic data demonstrate that hydrolysis and synthesis of protein cannot, under physiological conditions, be simple reversions of the same reactions. A "steady state" does exist *in vivo,* and this state is in the direction of synthesis, far from the thermodynamic equilibrium, in spite of an abundance of hydrolytic and oxidative enzymes demonstrably in operation. These facts compel us to envisage anabolic as well as catabolic reactions continually in operation even in the starving animal, the balance between them being maintained through the intervention of other reactions.

In the classical theory there is a "wear and tear" breakdown with little or no synthesis. According to the new theory, active resynthesis of protein occurs even in the starved animal. The direct proof was furnished by Tarver & Schmidt (3). There are many examples of breakdown of one tissue accompanied by synthesis of protein in other organs (14, 45, 46, 47).

It was predicted from the new theory that the protein-sparing action of carbohydrate occurs in the well-nourished animal as well as in the starving animal. This prediction was verified experimentally in the dog by Larson & Chaikoff (48) and in man by Cuthbertson *et al.* (49). Larson & Chaikoff observed in dogs, in nitrogen equilibrium, a retention of nitrogen when extra glucose was given at or nearly the same time as the protein. When the extra glucose was withdrawn, the stored nitrogen was slowly excreted. This experiment was a crucial test of the new theory. According to the classical theory, no storage was to be expected because in a well-fed animal in nitrogen equilibrium, the "wear and tear" quota is supplied by the diet and the remainder of the dietary nitrogen is the exogenous quota and is quickly catabolized.

It will be seen from the foregoing that the new theory accounts for all the experimental facts which are not in accord with the classical theory, as well as those employed to support it. Limitation of space does not permit discussion of other facts and phenomena cited in support of the classical theory. None are in conflict with the new theory and some are explained more easily by it.

The new theory of protein metabolism leads to a biological conception of the organism as a dynamic system in contrast to the mechanical nineteenth century concept of an adult organism as a machine which maintains its structure static except for a small change in "wear and tear." Within the concept of the organism as a dynamic system such phenomena as the formation of antibodies, adaptive hypertrophy and atrophy of muscles, and the influence of carbohydrate metabolism on protein metabolism take their place naturally and without strain.

Among the new problems which the new theory of protein metabolism has brought to the foreground are those concerning regulatory mechanisms—of the different capacity for variation in size and protein content of different organs, of the phosphocreatine content of muscle, of the constancy in concentration of free amino acids in the blood and tissues regardless of the nature of the diet.

Metabolism of Protein and Amino Acids in General

Cox *et al.* (50) found that an enzymatic digest of casein may be as effective as milk protein in promoting nitrogen retention while nitrogen equilibrium was not obtained with an acid hydrolysate of casein deficient in tryptophane. But even the latter incomplete source of nitrogen was more advantageous than no dietary nitrogen.

Goettsch *et al.* (16) found that after the intravenous or oral administration of an enzymatic hydrolysate of casein to hypoproteinemic dogs there is a smaller and slower rise in plasma-free amino nitrogen but this level remains above normal longer than in the normal dog. At the same time, there is less excretion of urea, amino acids, and ammonia in the urine of the hypoproteinemic animal in whom blood urea is also lower.

Shipley (20) found that the lowered nitrogen excretion and increased ketosis, which occur in the normal rat on fasting after a low protein diet, occur also in the hypophysectomized animal.

Waelsch reported previously (51) that in rats either fasted or placed on a protein-free, fat-carbohydrate diet there is an immediate

decrease in the urinary excretion of keto acids. This study was extended to the effects of feeding a series of amino acids to rats on a protein-free diet (52). Of twelve amino acids fed in the form of natural isomers, only two, tyrosine and lysine, gave definite increases of urinary keto acids. Glycine, glutamic acid, leucine, alanine, histidine, arginine, aspartic acid, and cystine were negative (the names of two others were not stated). The *dl*-forms of phenylalanine, methionine, isoleucine, valine, serine, threonine, aminobutyric acid, and phenylaminobutyric acid were positive, the intensities of response being in the order given.

Waelsch & Rittenberg reported further studies with N_{15} on the metabolism of glutathione (53). After feeding isotopic ammonia and *dl*-glutamic acid to rats, they found the isotope to be incorporated much more extensively into the glutathione than into the tissue proteins. Half of the glycine and glutamic acid in liver glutathione is replaced in four hours.

Metabolism of Individual Amino Acids

Rose & Fierke (54) have found aspartic acid and glucosamine to be dispensable components of food.

One of the important mechanisms for the conversion of methionine sulfur to cysteine sulfur has been established by independent and complementary studies of du Vigneaud and his co-workers, and of Stetten. Du Vigneaud *et al.* (55) prepared the *ll*-isomer of S-(β-amino-β-carboxyethyl)-homocysteine, $HOOC \cdot CH(NH_2) \cdot (CH_2)_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, and found that it can replace cystine in support of growth. It is incapable of supporting growth on a methionine-free diet containing choline. It is not, therefore, split to homocysteine. The authors suggested that this compound may be cleaved *in vivo* to give cysteine.

Brinkley & du Vigneaud (56) observed that *dl*-homocysteine and *dl*-serine shaken anaerobically with rat liver slices gave cysteine (determined as cystine). The *l*-isomer only was formed. The *d*-isomers of homocysteine and serine were ineffective. Pyruvic acid and ammonia, possible precursors of aminoacrylic acid, suggested as a possible acceptor of methionine sulfur (57), gave negative results. Methionine gave little or no extra cysteine; the demethylation of methionine is a necessary preliminary, therefore, to the transfer of methionine sulfur to form cystine.

Stetten (58) fed serine labelled with N^{15} to rats for three days

in small amounts. The very high isotope content of the cystine in the tissue proteins, five times as high as that found in glutamic acid and nearly equal to that found in the serine of the proteins, proved that the carbon skeleton of serine was converted to cystine.

Tarver & Schmidt (3), using radioactive sulfur, found the conversion *in vivo* of methionine sulfur to cystine sulfur to be a fast process, a finding in accord with those of du Vigneaud *et al.* and of Stetten.

Hess & Sullivan studied the effects of various amino acids on the excretion of cystine in two cases of cystinuria (59). In one subject, cysteine gave a slight increase, and glycine and methionine, no increase in urinary cystine; a high protein diet was definitely positive. In the second case, positive results were obtained not only with methionine, cysteine, and glutathione, but also with alanine, glycine, and glutamic acid. The increases with alanine were as great as those obtained with methionine. Cystine, in accord with results obtained by other workers, was consistently negative.

The authors ascribe the result with the nonsulfur-containing amino acids to a stimulation of "endogenous" metabolism although the total sulfur excretion was not increased with alanine or glycine and was decreased with glutamic acid; yet the urinary cystine was increased with all three. The increases of urinary cysteine after feeding cysteine and methionine were not much greater than those obtained with the nonsulfur-containing amino acids. Why not ascribe their effects also to a stimulation of "endogenous" metabolism?

In view of the findings of du Vigneaud *et al.* and Stetten (see above), it is possible that the occasional failure of methionine to increase the urinary cystine in a cystinuric may be due to an insufficiency of serine in such an individual. The positive result with a high protein diet in a subject negative to methionine, described above, is in accord with this suggestion.

Hess & Sullivan (60) obtained free cystine in a case of canine cystinuria only after precipitating the urine with cuprous chloride which disrupted a cystine complex and liberated the free amino acid. With this technique, they found that increasing the protein in the diet (casein) increased the cystine excretion. The increase in cystine output was greater proportionally than the increase in protein intake, especially at the lower protein levels. Similarly, methionine and cysteine gave greater increases of urinary cystine when added at lower than at higher protein levels.

Liver contains an enzyme capable of producing hydrogen sulfide

from cystine [Smythe (61)]. A procedure for concentrating and stabilizing the enzyme is described. Activity in the kidney, muscle, and brain is negligible. More hydrogen sulfide is produced under anaerobic than aerobic conditions.

Cystine also yielded hydrogen sulfide; glutathione and *dl*-homocystine were much less active; methionine and thioglycollic acid were inactive. Only 66.6 per cent of the cysteine was accounted for as hydrogen sulfide. Ammonia and pyruvic acid were found among the products of the reaction, suggesting but not proving the following reaction mechanism:

$$CH_2\,SH \cdot CH\,NH_2 \cdot COOH \rightarrow CH_2 = CNH_2 \cdot COOH + H_2S,$$

the aminoacrylic acid being split to pyruvic acid and ammonia. The reaction suggested for cystine is $RSSR + R_2S \rightarrow RRSH + S$. Sulfur and sulfhydryl groups were demonstrated in the reaction mixture.

Using radioactive sulfur, Smythe & Halliday (62) reported the presence of an enzyme in liver capable of converting sulfide to cysteine sulfur. The mechanism was not determined.

Blaschko (63) obtained from dog liver an extract capable, under anaerobic conditions, of decarboxylating cysteic acid to taurine.

The combination of arginine, glycine, chondroitin, and cystine constitutes the "cartilage growth factor." This combination gives results similar to those obtained with cartilage in support of growth and prevention of gizzard erosion (64).

Acetylglycine, formylglycine, and propionylglycine can serve as sources of glycine in the rabbit (65). When fed with benzoic acid they increase the amount of hippuric acid excreted.

Hamilton (66) has demonstrated the presence in dog and in human plasma of a substance which gives reactions similar to those of glutamine. The estimated concentration is 5 to 10 mg. per cent.

Leuthardt (67) reported the formation of hippuric acid from benzoic acid and glutamine by guinea pig liver slices. Rat liver slices are much less, if at all, effective. Asparagine, glutamic acid, and serine were positive but less effective with guinea pig slices than glutamine. Only the product of the condensation of benzoic acid and glutamine was identified as hippuric acid (see below).

Remmert & Butts (68) found that *l*(—)-histidine is a glycogen-former (in the rat) and accordingly also reduces induced ketonuria.

Featherstone & Berg (69) confirmed these findings. The deposition of glycogen was at approximately the same rate with *l*(—)-histi-

dine as with equivalent amounts of *l*(+)-glutamic acid. Since histidine is known to be degraded to glutamic acid (114), the authors suggest that their data admit the possibility that *l*(+)-glutamic acid is formed as an intermediary in the formation of glycogen from histidine. Glycogen formation from *d*(+)-histidine was less than from the *l*(−)-isomer and of doubtful statistical significance.

Darby & Lewis (70) studied the relation of urocanic acid to histidine metabolism in the rabbit and concluded that urocanic acid is probably not on the main pathway of histidine degradation, which is in accord with the observations on histidine metabolism noted above. Urocanic acid was isolated from the urine in half the animals when histidine was given orally; none after subcutaneous injection. Forty to seventy per cent of subcutaneously injected urocanic acid appeared in the urine. Only after large peroral doses of the acid did any appear in the urine. Severe toxic signs, in some cases fatal, appeared in all the animals excreting urocanic acid after receiving histidine by mouth. The authors concluded from the absence of toxic signs in all animals failing to excrete urocanic acid after histidine feeding that these animals did not convert a significant amount of the histidine to urocanic acid.

Almquist & Mecchi (71) estimated the *l*(+)-lysine requirement of the chick for optimal growth as approximately 0.9 per cent of the diet.

The sodium salt of the α-keto acid derived from methionine (α-keto-γ-methiolbutyric acid) is capable of replacing *dl*-methionine for growth purposes in young rats [Cahill & Rudolph (72)].

Tarver & Schmidt, using radioactive sulfur, demonstrated in dogs and rats that at least some methionine sulfur passes to taurine; this finding removes previous uncertainty on this point (73). The findings of du Vigneaud *et al.*, Stetten, and Blaschko, discussed above, suggest the following pathway: methionine → homocysteine (+ serine) → cysteine → cysteic acid → taurine.

Leuthardt & Glasson (74) demonstrated the formation of a small amount of glycine from serine. Hippuric acid was formed by the guinea pig liver slices in the presence of benzoic acid and serine. They confirmed the observations of Borsook & Dubnoff (75) that the product formed by rat liver slices from benzoic acid and serine is not hippuric acid.

Large doses of *dl*-serine are fatal to male rats [Fishman & Artom (76)]. The pathological findings are described in this same paper.

Borchers *et al.* (77) found that feeding *d*(+)-, *dl*-, or *l*(−)-tryp-

tophane to fasting rats was followed by a slight decrease in acetonuria, and by a marked decrease in the acetonuria induced by feeding sodium butyrate. But the tryptophane did not increase the liver glycogen. The authors concluded that although tryptophane is not ketogenic and does lower the acetone body excretion arising from administered sodium butyrate, the latter effect cannot be ascribed to formation of liver glycogen.

Rose *et al.* (78) have elucidated the fate of the carbon skeleton of valine. After injecting the *l*(+)-, *dl*-, or *d*(−)- forms of the amino acid to phlorhizinized dogs, three of the five carbon atoms were accounted for as extra urinary glucose. The result with *d*(−)-valine was unexpected because it is not interchangeable with *l*(+)-valine as an indispensable amino acid for growth (in the rat). α-ketoisovalerate also yielded three of its five carbon atoms as extra urinary glucose. Diminished acetone excretion was observed also, thus completing the proof that valine is an antiketogenic amino acid. These experiments indicate that in the diabetic dog valine is deaminized to the keto acid which then is converted to glucose.

Urea Formation

There is increasing expression of doubt regarding the ornithine cycle. The strength of the Krebs' theory (79) is based on the following established facts. Ornithine catalyses urea formation from ammonia by either liver slices or the intact perfused liver (80). Isotope studies (81, 82) showed that in this process carbon dioxide is taken up. When animals were fed isotopic nitrogen the concentration of the isotope found in the amidine group of arginine in the liver proteins was higher than in any other radicle containing transferred nitrogen (83). Arginine is rapidly hydrolyzed to ornithine and urea by liver slices. Finally there is the circumstantial evidence that arginase is found only in the livers of those animals whose main end product of nitrogen metabolism is urea (84).

Although the uptake of carbon dioxide as such was proven by isotope studies, it was not proven that the carbon dioxide was added directly to the ornithine and with ammonia thereby forming citrulline. Another explanation, and one which the free energy relations favor (see below), is that the carbon dioxide is first incorporated into a metabolite and that this substance, in giving up carbon dioxide for urea formation, may provide the free energy necessary.

The evidence with N^{15} is not unambiguous. The experimental data

show that all the very labile nitrogen components of protein had a high isotope content, they were all significantly lower than urea, and the amidine nitrogen of arginine did not occupy a preeminently unique position in this respect.

Schoenheimer *et al.* who cited their evidence as supporting the ornithine cycle theory pointed out another difficulty. Arginase acts only on free arginine, not on arginine in protein linkage. The isotopic arginine was isolated from liver protein. If the high isotope concentration in its amidine group is to be cited in support of the Krebs' theory, then

> it would be necessary to assume either that the arginine in protein linkage is involved in urea formation, or that free arginine and protein arginine continuously exchange their places in the liver.

Even if the ornithine cycle as originally formulated is correct as far as it goes, it is incomplete. The formation of urea from ammonia and carbon dioxide under physiological conditions entails a gain of nearly 14,000 calories of free energy per mol of urea (44) regardless of what the mechanism is. Reactions yielding the necessary free energy must therefore participate, with the probable effect that the ammonia and carbon dioxide do not enter into the cycle as such. The observed inhibition of urea formation from ammonia by oxidative poisons is to be expected from the necessity of introducing free-energy-yielding oxidation reactions into the cycle. Fitting into the same context is the catalytic effect of certain non-nitrogenous compounds, especially pyruvate, lactate (79), and oxalacetate (85). Leuthardt & Glasson suggested that the carbon dioxide may be carried into the cycle via oxalacetate, the oxalacetate being formed from pyruvate by the addition of carbon dioxide (86). Against this suggestion is the fact that oxalacetate is not always more effective than pyruvate even when no carbon dioxide is added to the medium, and is never markedly more effective.

The first serious doubts regarding the place of citrulline in the cycle were raised by Bach (87) who observed that when citrulline was used (in large concentrations) in place of ornithine, the ratio of urea nitrogen formed to ammonia which disappeared was one instead of two as expected from Krebs' theory. (This observation was confirmed in the reviewers' laboratory.) A catalytic effect of citrulline in low concentrations has not been recorded in the literature.

Trowell (80) confirmed the catalytic effect of ornithine in perfused rat livers, but observed that ammonia and ornithine often yielded

larger amounts of urea than equivalent amounts of arginine and that both were superior to ammonia plus citrulline. When a catalytic effect of a high concentration of citrulline was observed, it was only transitory, whereas that of ornithine persisted for several hours. It was observed further that adding ammonia in the presence of large amounts of arginine often accelerated urea formation. This result is difficult to understand if ammonia must pass through arginine in order to be converted to urea because the increase in the concentration of arginine effected by the ammonia must have been small. From the latter observation, Trowell suggested that the function of arginase in liver may be the provision of free ornithine and the main path of urea formation from ammonia does not pass through arginine.

In accord with this view is the observation of Bach & Williamson (88) that although a high concentration of ornithine inhibits the hydrolysis of arginine by rat liver slices, it does not inhibit urea formation from ammonia in the presence of lactate.

Circumstantial evidence casting doubt on the significance of citrulline in the cycle is the fact that the mechanism whereby citrulline is rapidly converted to arginine is strongly present in the kidney but is entirely absent from the liver (89).

Leuthardt (90) has adduced evidence of another mechanism which involves glutamine and asparagine, but not ornithine.

The possibility that glutamine may act as a nitrogen and carbon dioxide carrier in the formation of urea was suggested by Bach (87). This was not confirmed by Leuthardt (91) nor by ourselves (36).

Recently Leuthardt & Glasson suggested that the whole amide group of a postulated half-amide of oxalacetic acid may be transferred to ornithine, thereby forming citrulline. This suggestion is based on the observations that succinamide in the absence of ornithine is more effective than ammonia alone or ammonia plus succinic acid. No observations in the presence of ornithine were reported, which are necessary to determine whether ornithine is involved. We could not observe any evidence of the transfer of the amide group of glutamine to ornithine (36).

Labile Methyl Groups and the Related Metabolism of Choline and Its Derivatives

Meyer & du Vigneaud (92) found in the rat that only choline, lecithine, phosphoryl-choline, betaine, and dimethylethylhydroxyethyl ammonium chloride can serve as methyl donators.

On the other hand, in addition to the above five, the following compounds possessed lipotropic activity (i.e., prevented fatty liver): arsenocholine, alanine betaine, the aldehyde and acetal of betaine, homocholine, triethylcholine, cystine betaine, diethylmethylhydroxyethyl ammonium chloride, dimethylthetin, S-ethyl cysteine, S-methylcysteine, methionine, and its sulfoxide.

Almquist & Jukes (93) found that although arsenocholine is ineffective in the chick as a methylating agent, it is an effective substitute for choline as an antiperotic and for the promotion of growth.

Welch & Landau (94) showed that arsenocholine may, to some extent, replace choline in the biosynthesis of phospholipids, an observation which is in accord with its lipotropic action, and this in turn with the observations that choline stimulates the turnover of phospholipids (95) and is used in the synthesis of phospholipids (94). From these and other observations, Welch & Landau concluded that the lipotropic action of choline resides in the action of the intact molecule although its methyl groups are labile.

Jacobi & Baumann (97) and Stetten (98) have adduced evidence of the existence of another "essential" methyl donor in addition to methionine, choline (and betaine). Stetten's evidence is the most direct. Ethanolamine is methylated to choline at an unslackened rate whether the known methyl donors are present or not. Even when the body's store of labile methyl groups residing in methionine and choline is depleted by feeding large amounts of glycocyamine, which is irreversibly methylated to creatine (99) on a diet low in methionine and devoid of choline, there is no reduction in rate. The methylation of ethanolamine proceeds unchecked even when the degree of deprivation of the known methyl donors is so low as to produce fatty liver and renal hemorrhage. Even when choline and methionine are deficient in the diet, the body tends to maintain the concentration of choline constant by methylating ethanolamine. An insufficiency of ethanolamine is unlikely because it is formed from the reduction of glycine (100).

The lowered lecithine and choline content of the livers of rats on a diet deficient in lipotropic substances is accounted for not by a reduced rate of synthesis of choline but by its increased destruction [Stetten & Grail (101)].

Jacobi, Baumann & Meek (102) had observed earlier that the choline content of growing rats may increase four- to fivefold on a choline-free diet and that the choline content per gram of rat tissue remains constant on diets of varying composition (but containing methionine).

The same workers reared rats to the second generation on a diet free of choline but adequate in methionine. The tissues of the second generation contained only slightly less choline than rats on a diet containing adequate amounts of choline.

Jacobi & Baumann (97) showed that although choline prevents the renal hemorrhagic lesion in young rats produced by a diet low in choline and methionine, the specific biochemical defect in the kidney is not of choline because the concentration of the latter substance is not lowered in disease and may be even higher than in the healthy kidney in animals of the same age. Nor is the biochemical defect one of methionine because it is prevented and cured by choline on a diet so low in methionine that growth is not maintained.

From these and related experimental observations Jacobi & Baumann conclude that "The biochemical defect in rats with hemorrhagic kidneys is not in the choline content but rather in some other methylated essential."

Although du Vigneaud *et al.* (103) established with the use of deuterium that the methyl group of methionine may appear in choline, it has not been established that the mechanism of this reaction is a simple remethylation of demethylated choline. In fact, there is evidence to the contrary. Stetten (96) showed with N^{15} that when choline is fed, it is slowly, if at all, demethylated to ethanolamine, that although choline can undoubtedly serve as a biological source of methyl groups, ethanolamine is not the product of choline.

The labilization of the methyl groups of choline may involve a prior oxidation of the choline (97). The explanation suggested by Jacobi & Baumann for the renal hemorrhage when the diet is low in choline and methionine is that the extra fat which accumulates in the liver inhibits choline oxidase (104). As a consequence, the methyl groups in the choline become less available for methylation purposes owing to a failure in the necessary preliminary oxidation. Thus a deficiency in effective methyl donors results, including the unknown which Jacobi & Baumann postulate may be needed for maintenance of normal kidney structure.

Differing with this view, Landau & Welch (105) suggested that because arsenocholine and triethylcholine are lipotropic and prevent renal hemorrhage but are not methyl donors, renal hemorrhage is caused by a failure of phospholipid formation. Patterson & McHenry (106), who favor this view also, observed that in the absence of lipotropic substances in the diet the concentration of phospholipid was

less in rat kidney. This finding differs from that of Jacobi & Baumann although there was no significant difference in the total amount of phospholipid (the kidneys being larger in the diet deficient in lipotropic substances). The difference in concentration, though significant, was not great.

The discrepancy in the action of arsenocholine and triethylcholine can be reconciled as follows with the suggestion of Jacobi & Baumann that a methyl deficiency is the cause of renal hemorrhage. These two substances being lipotropic permit the oxidation in the liver of choline of which there is always an abundance, and as a result adequate amounts of the necessary methyl donor are available.

The reviewers have observed (36) that the methylation of glycocyamine by rat liver slices, in which the methionine has been proven to participate, is inhibited by the oxidative poisons: potassium cyanide, arsenite, and arsenate.

There are two ways in which an oxidative reaction could participate in the methylation of glycocyamine by methionine. One is that an oxidation product of methionine and not methionine itself is the actual methylating agent; but the sulfoxide, sulfonium, and keto acid derivatives of methionine failed to relieve the effect of the oxidative poisons (36). It was found with tissue slices that the sulfoxide but not the sulfonium compound could replace methionine as a methylating agent, findings in accord with the respective ability and failure of these compounds to replace methionine for growth (107, 108).

In the second possible mechanism methionine participates only indirectly in the methylation of glycocyamine; the direct methylating agent is an oxidation (fully methylated) product of choline and the effect of the methionine is to restore the fully methylated oxidation product of choline but not of choline itself. Hence there is the appearance of the methyl groups of methionine in creatine.

In the experiments in which the participation of methionine in creatine formation was established, it was found that choline was ineffective. This is not inconsistent with the second possible explanation given because there was certainly an abundance of choline already in the liver slices. The extensive methylation of glycocyamine which liver slices effect without added methionine could be accounted for by the oxidation of pre-existing choline.

Stetten & Grail (101) made the interesting observation that cystine greatly increases the lipid content of the liver and at the same time leads to a marked increase in the choline and phosphorus of the liver

lipids and to a lower N/P ratio than is obtained whether choline is present in the diet or not. They concluded that these results

> are incompatible with any simple concept of antagonism between choline and cystine with regard to liver lipid, and their relationship to the current explanation of fatty liver in choline deficiency is not clear.

The same results as with cystine were obtained with homocysteine. Yet Stetten & Grail suggest that the failure of homocysteine as a methyl acceptor to reduce the choline content of rat tissues is because homocysteine is methylated to methionine and thereby serves as a carrier of methyl groups (to form choline presumably). A more probable explanation appears to lie in the observations of Brinkley (56) and of Stetten himself that homocysteine and serine form cysteine; then either the cysteine thus formed or the cystine derived from it exerts the same influence on liver lipids as when cystine is fed.

Treadwell *et al.* (109) observed that free methionine exerts its lipotropic action in the absence of other protein (casein) constituents, contrary to the findings of Channon *et al.* (110) and of Best & Ridout (111). Their data suggest that the free amino acid exerts a greater lipotropic action than when given in equivalent amounts as casein.

Daft *et al.* (112) studied the etiology of the liver damage which appears in rats on a diet low in protein (casein sole source). They found that added cystine increased the cirrhosis, but prevented hemorrhage or necrosis. Methionine and choline prevented the whole syndrome of cirrhosis, hemorrhage, and necrosis. The authors concluded that cirrhosis on the one hand and hemorrhage plus necrosis on the other are the results of two separate etiological entities.

Sprunt (113) found that choline, methionine, and betaine increase the rabbit's resistance to inoculated vaccinia. Choline and methionine were equally effective and three to four times as effective as betaine. Glycine and cystine were ineffective. The protective action was ascribed to the methyl groups in the substances which were effective.

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THE CHEMISTRY OF THE CARBOHYDRATES

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Introduction

In order to review as comprehensive a subject as carbohydrate chemistry within the space available, it becomes necessary to restrict the treatment to a limited number of the fields involved. Inasmuch as comprehensive reviews on starch (1), cellulose (2), lignin (3), pectins, and other polysaccharides (4) have appeared in this and other publications in the last three years, the present chapter of the *Review* has been devoted for the most part to the chemistry of the sugars and their derivatives. The natural glycosides have not been considered because their biological properties for the greater part spring from aglycon groups which are of primary interest to reviewers of other subjects (5). The phosphoric esters have been omitted because they were treated in detail in Volume XI of the *Annual Review of Biochemistry* (6).

In Volume IX of the *Annual Review of Biochemistry* the present reviewer covered recent developments in carbohydrate chemistry with particular emphasis on the stereomeric factors affecting the course of reactions. Although this phase of carbohydrate chemistry has continued to develop, it has not been stressed in the present discussion. Rather, emphasis has been placed upon the effect of unsaturation in directing and influencing carbohydrate reactions. Emphasis on this phase of the subject seems of special importance in a biological review because many substances which play important parts in biological reactions contain unsaturated groups capable of supplying, removing, or transporting electrons from one component of the system to another by processes of electron displacement (7, 8). In order to develop the subject from a unified viewpoint in the present review, the liberty has been taken of interpreting a number of reactions in terms of electron displacement, even though they were not so interpreted in the original publications. It is not intended to give the impression that all or even most reactions involving unsaturated groups can be explained by any one mechanism, but rather to clarify and correlate some reactions

which have been considered unusual, and to provide a stimulus for further investigation.

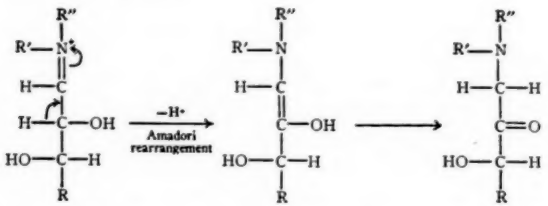
For some years there has been a lull in the production of needed books in English on the chemistry of the carbohydrates. It is, therefore, gratifying to record the appearance of a number of noteworthy new reference and textbooks (9).

Nitrogen Glycosides and Related Compounds

Inasmuch as certain biologically important compounds contain sugar residues in glycosidic linkage with nitrogen, the properties of compounds of the N-glycoside type are of particular interest to the biochemist. For this reason, many nitrogen glycosides have been prepared and carefully studied (10); they differ from the oxygen glycosides primarily in their capacity to react reversibly with hydrogen ions, and to form systems capable of rearrangement and of initiating oxidation-reduction changes. In contrast to the oxygen glycosides, the nitrogen glycosides give mutarotation reactions (11) catalyzed by water, hydrogen chloride, and other substances. The mutarotation reaction may take place in the nitrogen glycosides of primary amines both through the Schiff base and through its cation, but in glycosides of secondary amines the reaction is possible only through the cation.

Schiff base,
α-N-glycoside ⇄ or its cation ⇄ β-N-glycoside

Amadori rearrangement.—The cation of the Schiff base (I) readily undergoes a rearrangement, discovered by Amadori and clarified by Kuhn & Weygand (12).



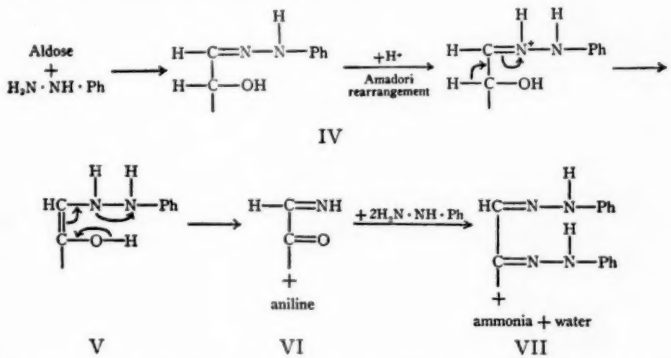
I. Schiff base cation[1] II. Sugar enol III. Ketose

[1] In accordance with usual practice (8), the curved arrow is used in this review to denote the direction of movement of an electron pair.

The reaction which is induced by the positive charge on the nitrogen may be considered to consist of a flow of electrons toward the nitrogen, accompanied by the liberation of a proton from carbon 2 (I) to yield the substituted sugar enol (II). The latter is a derivative of a ketose and establishes equilibrium with other modifications of the sugar in the well-known manner.

The Amadori reaction thus provides a convenient method for preparing N-substituted ketoses ("arylisoglycosamines"), from aldoses (13). The derivatives prepared by treatment of aldoses with aromatic amines are of commercial importance because on reduction they yield epimeric "1-arylglycamines" (1-arylamino-1-desoxysugar alcohols), some of which are important intermediates for the preparation of flavins. By selection of suitable conditions, either epimer can be produced in predominating quantity. Thus, D-ribamine, useful for the synthesis of vitamin B_2, can be obtained from D-arabinose by means of an Amadori rearrangement followed by reduction of the resulting "isoarabinosamine."

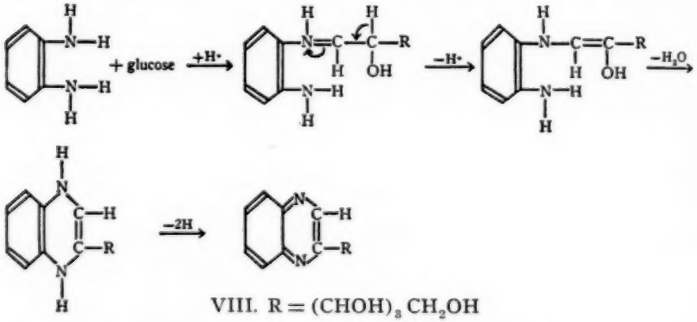
Osazone formation.—The facility with which "arylisoglycosamines" yield osazones lead Weygand (14) to suggest that osazone formation might include an Amadori rearrangement. Although several routes for the conversion were considered possible, the following diagram illustrates the general principle:



The cleavage of the hydrazo compound (V) to aniline and an iminoketone (VI) provides a means for the oxidation of the second carbon without assuming direct oxidation by phenylhydrazine, a step previously assumed but nevertheless improbable. The cleavage reaction

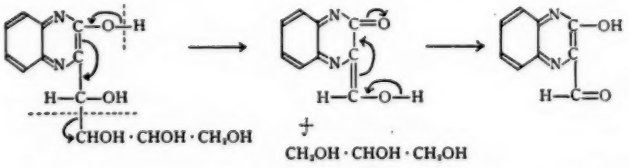
may follow a course such as that indicated by the arrows of the formula (V). However, since the observation has been made (15) that osazone formation can be inhibited by careful elimination of oxidizing agents, the possibility of a free radical mechanism should be investigated.

Quinoxaline derivatives.—The ability of the nitrogen glycosides to form systems capable of rearrangement and of initiating oxidation-reduction reactions is illustrated further by the properties of the condensation products derived from *o*-phenylenediamine. It has been known for years that evaporation in air of a solution of glucose containing *o*-phenylenediamine yields 3-(D-*arabo*-tetrahydroxybutyl)-quinoxaline (VIII) in addition to other products. An Amadori rearrangement has been suggested in explanation of the formation of the quinoxaline derivative (13).



VIII. R = $(CHOH)_3 CH_2OH$

Since quinoxaline derivatives are formed more readily by use of a mild oxidizing agent, a mechanism involving oxidation of the sugar to the osone has been advanced in place of the Amadori mechanism (16). When 2-hydroxy-3-(D-*arabo*-tetrahydroxybutyl)quinoxaline (IX) is heated in water with phenylhydrazine (17), it is split to

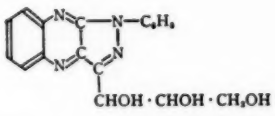


IX X XI

glycerol and 2-hydroxyquinoxaline-3-aldehyde (XI) (isolated as the phenylhydrazone in 80 per cent yield). Likewise, 2-phenyl-3-(D-*arabo*-tetrahydroxybutyl)quinoxaline and 3-(D-*arabo*-tetrahydroxybutyl)quinoxaline show cleavage to the 3-aldehyde, but the reaction is much slower than in the case of the 2-hydroxy compound (18).

To account for the rapid cleavage of the hydroxy compound, the reviewer suggests that removal of a proton from the hydroxyl at carbon *2* is followed by the electron flow indicated by arrows in the formula (IX). This results in cleavage of a glycerol fragment which combines with a proton. Subsequently, a flow of electrons in the reverse direction, accompanied by proton addition and cleavage (X), regenerates the quinoxaline structure (XI). This mechanism would involve a reversible electron change accompanied by a reversible proton change, a process which might be applicable to certain enzymatic cleavages.

The cleavage reaction of these quinoxaline derivatives seems to take place only in neutral or slightly alkaline solution. When the solution containing the tetrahydroxybutylquinoxaline is made acid, an entirely different reaction takes place with addition of phenylhydrazine and cyclization of the side chain to yield 1-phenyl-3-(D-*erythro*-trihydroxypropyl)flavazole (XII). Although an Amadori rearrangement was considered possible (19), the investigators favored a mechanism involving direct oxidation of the tetrahydroxybutylquinoxaline by the phenylhydrazine, followed by cyclization. An intermediate Amadori rearrangement would account for the fact that the reaction requires the presence of acid.

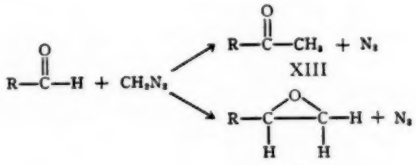


XII. 1-Phenyl-3-(D-*erythro*-trihydroxypropyl) flavazole

Reactions Initiated by Diazomethane

Reactions of aldehydo sugars.—In accordance with the mechanism proposed by Arndt & Eistert (20) to explain the behavior of simple aldehydes with diazomethane, it has been found that *aldehydo* sugars on treatment with diazomethane give two products (21). One of these

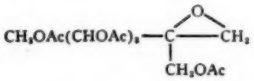
is a methyl ketone (XIII) and the other is an ethylene oxide derivative (XIV).



XIII

XIV

Treatment of *aldehydo*-D-arabinose tetraacetate with diazomethane gives 1-desoxy-*keto*-D-fructose tetraacetate (XIII) and an unidentified second product. Since the carbonyl group of a ketose carries no replaceable hydrogen, it cannot undergo the first reaction and the ethylene oxide (XIV) is formed. Treatment of *keto*-D-fructose pentaacetate with diazomethane yields a crystalline ethylene oxide derivative (22),



Reactions of aldonyl chlorides.—In contrast to the behavior of *aldehydo* sugars, acetylated aldonyl chlorides combine with diazomethane and form acetylated 1-diazo-1-desoxyketoses (23).

$$CH_2OAc(CHOAc)_nCO \cdot Cl + 2CH_2N_2 \rightarrow \underset{\text{1-diazo-1-desoxyketose acetate}}{CH_2OAc(CHOAc)_nCO \cdot CHN_2} + N_2 + CH_3Cl$$

$$RCO \cdot CHN_2 + HOAc \rightarrow RCO \cdot CH_2OAc + N_2$$

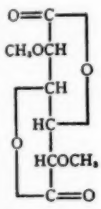
$$RCO \cdot CHN_2 + H_2O \xrightarrow{Ag_2O} RCH_2COOH + N_2$$

These compounds react with acetic acid to give open-chain ketose acetates, and with hydrogen chloride or bromide to give 1-chloro or 1-bromo open-chain ketose acetates. Furthermore, on treatment with silver oxide or other catalyst, the diazodesoxyketoses rearrange with loss of nitrogen to form 2-desoxyaldonic acids. This process is an application of the Arndt-Eistert synthesis, which has been thoroughly reviewed in a recent publication (24).

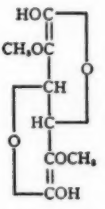
Reactions of acids and lactones.—The selective methylating action of diazomethane provides a simple means of esterifying the carboxyl

group of uronic acids without glycoside formation (25). Treatment of aliphatic lactones with diazomethane ordinarily opens the ring and gives a methyl ester with a free hydroxyl (26). Coumarin and diazomethane, however, give 3-carbomethoxy-4-(*o*-methoxyphenyl)-pyrazoline. The lactone ring is opened, both the carboxyl and the resulting phenolic hydroxyl are esterfied, and a third molecule of diazomethane adds to the ethylenic double bond to form the pyrazoline ring. This reaction has been applied as a test for the coumarin type of linkage in lignin.

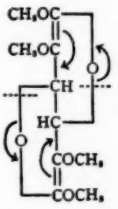
Although aliphatic hydroxyls do not usually react with diazomethane, the methylation of *alpha* hydroxyls of tartaric, trihydroxyglutaric, and saccharic acids by diazomethane has been known for several years (27, 28).



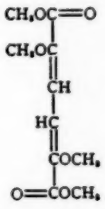
XV. 2,5-dimethylmannosaccharic dilactone



XVI. Enolic form of 2,5-dimethylmannosaccharic dilactone



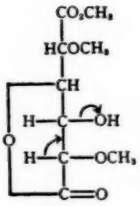
XVII. Methylated enolic form of 2,5-dimethylmannosaccharic dilactone



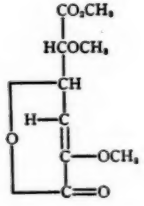
XVIII. Dimethyl ester of dimethoxymuconic acid

Recently, it has been found (29) that treatment of mannosaccharic acid, or its lactone, with two moles of diazomethane gives 2,5-dimethylmannosaccharic dilactone (XV). Further treatment, however, gives the dimethyl ester of diamethoxymuconic acid (XVIII). Schmidt & Kraft, who studied the reaction, concluded that the unsaturated compound results from the dimethyl dilactone through the following steps: cleavage of the lactone rings; methylation of the resulting carboxyl groups; and cleavage of water between carbon atoms 2 and 3, and 4 and 5. The reviewer, however, suggests an explanation based on the hypothesis that diazomethane replaces the enolic hydrogen of 2,5-dimethylmannosaccharic dilactone (XVI) with methyl groups in the usual manner. A flow of electrons in the resulting compound (XVII), as indicated by the arrows, gives the dimethyl ester of di-

methoxymuconic acid, actually isolated. This mechanism is supported by the fact that dimethylmannosaccharic dilactone combines with iodine in alkaline solution and gives other reactions characteristic of enolic compounds. The closely related substance, saccharic acid monolactone, however, on treatment with diazomethane (28) gives an unsaturated lactone ester (XX). The formation of this compound can be explained by a flow of electrons from the activated hydrogen of carbon 5 to the adjacent carbon-carbon bond, as indicated in the formula (XIX). The fact that the compound (XX) is obtained by the oxidative and methylative action of Purdie's reagent on 1,2-isopropylideneglucofururono-γ-lactone (30) shows that the reaction is not unique for diazomethane.



XIX

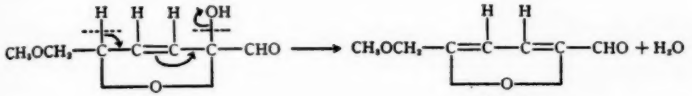


XX

Unsaturated and Aromatic Compounds Derived from Enolic Substances

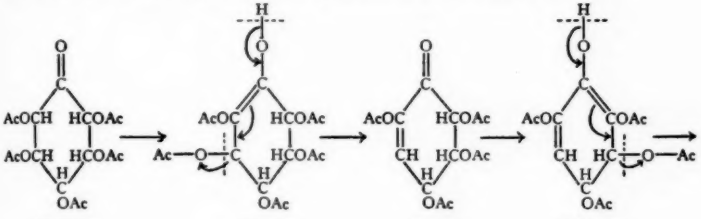
Formation of furfural derivatives.—A mechanism somewhat similar to that suggested for the transformation of enolic lactones to various unsaturated compounds can be applied to the formation of furfural and its derivatives from enolic forms of the methylated sugars and glycoseens. It has been known for some time that enols of methylated pentoses, on treatment with dilute acid in the cold, yield furfural (31). The mechanism of this process has been clarified by the preparation (32) of 5-(methoxymethyl)-2-furaldehyde (XXII) from tetramethyl-1,2-glucoseen through an intermediate 3,4-unsaturated osone (XXI). The formation of the latter substance may be explained satisfactorily by a series of successive electron displacements analogous to those noted in the preceding paragraph. The conversion of the unsaturated osone to the furfural derivative involves cleavage

of water which might be expected to take place as indicated in the formula (XXI).

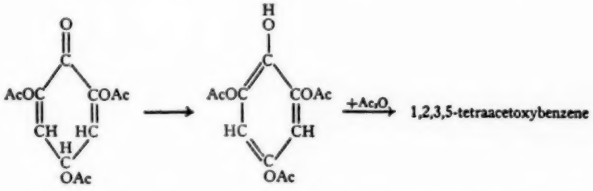


XXI XXII

Formation of benzene derivatives from inositol.—Inositol has long held interest as a possible connecting link between the carbohydrates and the naturally occurring aromatic compounds. Partial oxidation of *i*-inositol with *Acetobacter suboxydans* yields a monoketoinositol (33), which has a different configuration from the monoketoinositol (inosose) obtained by careful oxidation with nitric acid (34). Both compounds give crystalline *keto*-pentaacetates (XXIII), which on treatment with pyridine in the presence of acetic anhydride give 1,2,3,5-tetraacetoxybenzene. The investigator suggested that the reaction probably takes place through an enolic intermediate. A mechanism involving enolization and consecutive electron displacement is indicated in detail by formulas (XXIII to XXVIII).



XXIII XXIV XXV XXVI



XXVII XXVIII. 2-hydroxy-1,3,5-acetoxybenzene

Treatment of the monoketoinositolpentabenzoate with sodium acetate or pyridine in acetic acid gave a substance designated as

1-hydroxy-2,3,5-tribenzoxybenzene, but not rigorously identified. According to the mechanism advanced by the reviewer, a 2-hydroxy-1,3,-5-tribenzoxybenzene should have been formed. The nitric acid oxidation of inositol to a mixture of hydroxyquinones, commonly designated as rhodizonic acid, has been investigated and a series of esters and salts prepared (35). In the production of rhodizonic acid, the inositol appears to be oxidized to a triketo derivative which on enolization gives hexahydroxybenzene, a substance readily oxidized further by air (36). The extent of air oxidation depends on the experimental conditions, especially the acidity, temperature, and method of aeration. By the addition of potassium carbonate with short aeration, the potassium salt of tetrahydroxyquinone is formed, and by the addition of potassium acetate with prolonged aeration, potassium dihydroxydiquinone is obtained. The preparation of a diketo-*i*-inositol by the prolonged action of *Acetobacter suboxydans* on *i*-inositol has been reported (37), but its conversion to aromatic compounds has not been investigated.

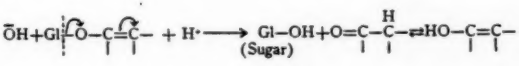
Alkaline Cleavage of Glycosides

Although several alkali-sensitive glycosides have been known for a long time (38), susceptibility to hydrolysis by acids and resistance to hydrolysis by bases have been considered to be characteristic properties of the glycosides in general. The alkaline hydrolysis of many glycosides has now been investigated and a number of generalizations can be made.

Several years ago, Evans & co-workers (39) pointed out that oligosaccharides containing the biosidic linkage Gl—O—C=C— should be susceptible to alkaline hydrolysis. In agreement with this concept, it was found that 3-glucosyl-fructose (turanose), is readily hydrolyzed by alkali, probably through the intermediate enediol, which form would have the unsaturated biosidic linkage (40). The existence of saccharides of known structure, readily cleaved by alkali, lends support to the idea that the changes in the viscosity of a solution of oxycellulose on treatment with alkali (41) are caused at least in part by alkaline hydrolysis of glycosidic linkages in the primary oxidation product. The oxycellulose obtained by periodate oxidation (42) contains aldehyde groups adjacent to the glycosidic unions and thus fulfills through enolization the unsaturation requirement for alkali-sensitive linkages. This material is, indeed, particularly sensitive to alkali

(43), but conversion of the aldehyde groups to carboxyl groups through oxidation with chlorous acid destroys the alkali sensitivity (44). A reasonable interpretation appears to be that carboxyl groups do not enolize readily, as do aldehyde groups, to yield the requisite unsaturated structure. Although saturated aliphatic glycosides are not hydrolyzed by alkali, a study of the behavior of aromatic glycosides with alkali has revealed that the rate of hydrolysis follows in general the acidity of the parent phenol and that for glycosides of strongly acidic phenols the catalytic effect of bases is as great as that of acids (45). The striking sensitivity of salicylic acid glucoside, which is hydrolyzed even by water at room temperature, led to the investigation of the *beta* glucosides of $(CH_3)_2C:CHCH_2CH_2CH(OH)CH_3$ and $CH_3CH(OH)CH_2CH_2CHO$ (46). Unlike salicylic acid glucoside, these compounds do not contain a point of unsaturation adjacent to the glycosidic union and are not hydrolyzed by alkali. It is thus apparent that the activating effect of the double bond does not extend through two or more intermediate saturated carbons.

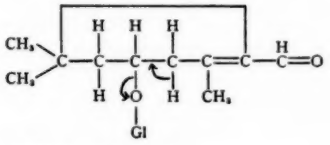
Theoretically, glycoside cleavage can occur on either side of the glycosidic oxygen. When the conditions are such that the aglycon group is able to take electrons (through the oxygen) from the sugar residue, cleavage of the sugar-oxygen bond is favored, but when the sugar residue is able to take electrons from the aglycon group, cleavage of the aglycon-oxygen bond is favored. Cleavage of the sugar-oxygen bond seems to occur during the alkaline hydrolysis of glycosides containing unsaturated groups in the aglycon adjacent to the glycosidic union. In these cases, alkaline cleavage appears to involve addition of a hydroxyl to the glycosidic carbon, accompanied by an electron shift in the aglycon and the addition of a proton as indicated below:



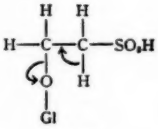
In the glucoside of salicylic acid, the process is facilitated by the capacity of the carboxyl group in conjunction with the aromatic nucleus to take up electrons reversibly.

Cleavage of the aglycon-oxygen bond appears to take place in the hydrolysis of picrocrocin, the bitter principle of saffron (47). The sensitivity of the substance to alkaline hydrolysis may be explained by

the following steps: activation by the conjugated double bond system of the hydrogen on the carbon adjacent to the glycoside-bearing carbon; removal of the hydrogen by means of the base; a subsequent electron shift indicated by the curved arrows in the formula (XXIX); and combination of the glucoside residue with a proton. The products should then be glucose and 2,6,6-trimethyl-5,6-dihydrobenzaldehyde (safranal), and, indeed, the latter substance was isolated from the hydrolyzate. Apparently the alcohol corresponding to the parent glycoside was not formed even as an intermediate product. The reviewer suggests a similar explanation for the alkali sensitivity of the 2-hydroxyethanesulfonic acid glycosides (48). The analogy may be seen from the formula (XXX) in which the hydrogen of the adjacent carbon can be assumed to be activated by the sulfonyl group.



XXIX. Picrocrocin



XXX

Anhydro Derivatives

Formation from phenyl glycosides.—In the preceding section it was pointed out that cleavage of glycosides with alkali may take place on either side of the glycosidic oxygen. Cleavage of the sugar-oxygen bond under suitable conditions might be expected to result in anhydro compounds, and, indeed, 1,6-anhydro derivatives have been obtained in good yield by heating phenyl glycosides with aqueous alkali (49). The reaction rate was found to vary widely for phenyl glycosides of different sugars and for *alpha* and *beta* isomers of the same sugar. The results so far reported indicate that anhydro derivatives are formed from *beta* phenyl glycosides more rapidly than from *alpha*. *Beta* phenyl hexopyranosides presumably have a *cis*-configuration for the phenyl group of carbon 1 and the hydroxyl of carbon 6. Hence the greater reactivity of the *beta* phenyl glycosides is not in accord with an opposite-face mechanism (50) for a single replacement such as that shown by the tosyl (*p*-toluenesulfonyl) derivatives. Further investigation may reveal the reason for the difference.

Formation from polysaccharides.—The pyrolysis of starch (51),

vegetable ivory (52, 53), lactose (54), and related compounds has made 1,6-anhydro derivatives of glucose, mannose, and galactose available for synthetic purposes. The isopropylidene derivative of 1,6-anhydro-β-D-mannopyranose ("D-mannosan"), with a free hydroxyl on carbon 4, can be used for the synthesis of 4-substituted derivatives of mannose (52, 55), and the isopropylidene derivative of 1,6-anhydro-β-D-galactopyranose ("D-galactosan"), with a free hydroxyl on carbon 2, can be used for the preparation of 2-substituted derivatives of galactose (56). Particularly noteworthy (55) is the preparation through anhydro derivatives of 4-(β-D-glucosyl)-D-mannose and 4-(β-D-galactosyl)-D-mannose, which were converted to cellobiose and lactose, respectively. "4-Tosyl-D-mannosan," upon treatment with aqueous sodium hydroxide, was detosylated with the formation of "3,4-anhydro-D-talosan ‹1,5›β‹1,6›," a very interesting substance possessing three rings (57). A similar compound was prepared from "2-tosyl-D-galactosan" (56).

Influence of the anhydro group on the primary ring structure.—It has been found that the presence of an anhydro ring influences in marked degree the tendency or ability of the sugar to exist in the customary pyranose and furanose modifications (58). In some cases, the molecule containing the furanose ring is even more stable than the one containing the pyranose ring. Thus the *alpha* and *beta* methyl pyranosides of 3,6-anhydroglucose, on contact with hydrogen chloride, change very rapidly to the corresponding furanosides. The authors point out that in a structural model of 3,6-anhydroglucose the hydroxyl groups on carbons 4 and 5 are so situated as to allow the formation of either a furanose or a pyranose ring, but that the pyranose ring is highly strained. In 3,6-anhydrogalactose, also, the pyranose ring is strained, but the hydroxyl at carbon 4 lies in such a position that it is unable to form a furanose ring without rupture or distortion of the anhydro ring (59). Experimentally, neither α- nor β-methyl-3,6-anhydro-D-galactopyranoside could be transformed to a furanoside. The strain induced in the pyranose ring by the 3,6-anhydro ring explains the existence of 2,4-dimethyl-3,6-anhydro-D-galactose in the *aldehydo* form, nothwithstanding the presence of a free hydroxyl on carbon 5. This tendency of 3,6-anhydrogalactose derivatives to exist in the free aldehyde modification was advanced (60) to account for the production of a 2,5-dimethyl-3,6-anhydro-L-galactose derivative from methylated agar.

Miscellaneous reactions yielding anhydro derivatives.—Other

unique reactions yielding anhydro compounds are those of 1,2,3-triacetyl-5-trityl-D-ribose with hydrogen bromide in acetic acid to give 1,5-anhydro-2,3-diacetyl-D-ribofuranose and trityl (triphenylmethyl) bromide (62), and of 1,2-isopropylidene-D-glucofuranose 5,6-carbonate on heating to give 1,2-isopropylidene-3,6-anhydro-D-glucose and carbon dioxide (63). A 3,6-anhydro-derivative was prepared from methyl 3-tosyl-4,6-dibenzoyl-2-methyl-α-D-altroside and called "2-methyl-3,6-anhydro-α-methylaltroside" although its configuration was not rigorously proved (64). According to the opposite-face mechanism discussed in a previous review (50), inversion should have taken place on carbon 3 with the formation of a mannose derivative. Likewise, cleavage of a 2,3-anhydro-D-mannoside with hydrazine hydrate should have given a 2-hydrazino-D-glucoside rather than the 2-hydrazino-D-altroside reported (64).

Esters

Sulfates.—The preparation of 3,6-anhydro compounds of glucose and galactose from 6-sulfates indicates that the cleavage of sulfates is similar to that of sulfonates (65).

A stimulating hypothesis has been presented (60) for the biological synthesis of the agar polysaccharide, based on the assumption that a sulfate fulfills a function similar to that of a phosphate in the enzymatic synthesis of starch. Supposedly, D-galactose-1-sulfate by elimination of the sulfate group forms a chain of ten D-galactose residues combined by 1,3-glycosidic linkages. The last member of the chain, bearing its sulfate group, undergoes an intramolecular change whereby the aldehydic carbon is reduced by the $-CH_2OH$ group of the same member. This results in a shift in the position of the reducing group so that the D-galactose-1-sulfate residue, linked by the 3-position, becomes an L-galactose-6-sulfate residue, linked by the 4-position. The L-galactose-6-sulfate residue, however, would have free hydroxyls on carbons 2 and 3 and would be capable of oxidation by periodic acid. Other work indicates that agar, supposedly containing the L-galactose-6-sulfate residue, is not oxidized by periodic acid (61); hence, further investigation seems to be in order.

Hydrolysis of carboxylic esters.—The hydrolysis of carboxylic esters ordinarily takes place with rupture of the acyl-oxygen bond. Evidence has now been obtained which indicates that the acid-catalyzed alcoholysis of carboxylic esters of tertiary alcohols proceeds, at least in part, by rupture of the alkyl-oxygen bond (66). The base-

catalyzed reaction, however, takes place in normal manner with rupture of the acyl-oxygen bond. An excellent discussion of the mechanism of ester cleavage has been given (67).

Orthoacetates.—In confirmation of the opposite-face mechanism for the formation of orthoacetates, it has been found that the acetylated glycosyl halides of neolactose and of α-guloheptose (D-glycero-L-talo-aldoheptose), on treatment with silver oxide and methyl alcohol, yield methyl orthoacetates (68). On account of the facility with which triacetyl - bromo - 3 - mesyl-glucofuranose (mesyl = methanesulfonyl) forms a methyl orthoacetate, an orthoester structure was advanced for the bromo compound (69).

Fluoro derivatives.—Heretofore there has been no means for replacing, either directly or indirectly, the primary hydroxyl groups of glucose and similar sugars with fluorine. Recently 6-fluoro derivatives have been obtained through the reaction of the corresponding mesyl derivatives with potassium fluoride (70).

Open-chain esters.—Methods have been worked out for the preparation of acetylated acyclic sugars containing acetyl, methoxyl, ethoxyl, mercaptal, chloride, bromide, or phosphate groups attached to the aldehydic or ketonic carbon (71). The acetal carbon of the mixed acetals as, for example, that of 1-methoxy-*aldehydo*-D-mannose hexaacetate is asymmetric and gives rise to two diastereomers which in some cases have been separated (72).

Propionates and benzoates.—Propionyl derivatives of sugars have been found to crystallize well and to be suitable for separation of sugar mixtures by distillation; the procedure has given good results in the analysis of corn sirups and other carbohydrate mixtures (73). Numerous azobenzoates and 2,4-dinitrobenzoates have been prepared as reference compounds (74); the azobenzoates are suitable for use in chromatographic separations (75).

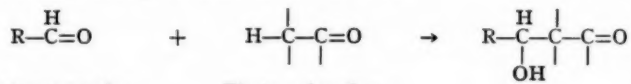
Carbonates.—Esters containing two sugar residues in combination with a carbonate radical have been obtained by reaction of sugar derivatives having one free hydroxyl, with carbonyl chloride in the presence of pyridine (76, 63). Attempts to prepare disaccharides by cleavage of carbon dioxide from mannose glucose carbonate were unsuccessful even though 1,2-isopropylidene-D-glucofuranose 5,6-carbonate yields carbon dioxide and a 3,6-anhydro compound on heating (63).

Thiocyanates.—By treating acetylglycosyl halides with silver thiocyanate, Fischer obtained acetylglycosyl isothiocyanates. True thiocyanates of sugars have now been prepared by the use of potassium

thiocyanate (77). In the presence of sodium methylate, thiocyanic esters decompose to disulfides whereas isothiocyanic esters yield thiourethans, $GlNH \cdot CS \cdot OCH_3$. On mild heating, thiocyanate groups attached to glycosidic carbons rearrange to isothiocyanates, but thiocyanate groups attached to primary carbons are stable. By treatment of methyl glycosides with phenyl isocyanate in pyridine solution, the alcoholic groups react in normal fashion to give phenylurethans, $PhNHCO \cdot OR$ (78).

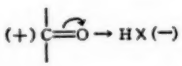
Reactions of the Aldol-Condensation Type

The aldol condensation is one of several, closely related reactions which depend on the unsaturation of the carbonyl group and its tendency to combine with electron-donating groups. These reactions have been represented by the general scheme (79):



Electron-accepting component (A) — Electron-donating-component (B)

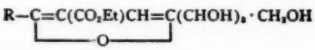
The condensation may be effected either by acidic or by basic catalysts. In condensations effected by acids, the electron-accepting component (A) is activated presumably through a co-ordination complex,



in which the electrons are displaced from the carbonyl carbon, leaving a transient carbonium ion capable of reaction with the electron-donating component (B). In condensations effected by bases, the electron-donating component is activated presumably through its enolate ion which acquires the resonance form, $:C-C=O$, as it approaches the electron-accepting component. Substances which yield electron-donating groups readily as, for instance, hydrogen cyanide condense with carbonyl compounds rapidly, and strongly basic catalysts are unnecessary. But substances which yield electron-donating groups less readily require strongly basic catalysts, and the rate of reaction is largely conditioned by the efficiency of the catalyst in causing incipient ioniza-

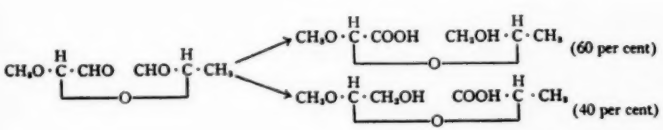
tion of the active hydrogen. Frequently, reagents other than acids and bases, as, for example, amino acids and compounds capable of yielding enediols, promote aldol condensations in a somewhat obscure manner (80). It seems probable that the enediols act through intermediate compounds which facilitate the formation of active radicals. It is noteworthy that the cleavage of 2-hydroxy-tetrahydroxybutylquinoxaline [discussed on page 208] is the reverse of an aldol condensation, and the means of electron transfer described there may be pertinent to the synthetic reactions induced by enediols.

Interest in the practical application of the aldol condensation is shown by the appearance of numerous patents (81) for the preparation of hydroxyaldehydes and related substances by condensation of aldehydes in the presence of basic catalysts. Several publications describe the condensation of sugars with substances containing active hydrogen in the presence of acidic catalysts. Phenol combines with glucose in the presence of hydrogen chloride to give *p*-hydroxyphenylsorbitol (82). The reaction involves addition of the aromatic nucleus and hydrogen to the carbonyl group of the sugar. Attempts to condense glucose and other sugars with enolizing ketones in the presence of zinc chloride have not been generally successful although crystalline compounds have been obtained with acetoacetic ester (83), with benzoylacetic ester, and with acetonedicarboxylic ester (84). In these cases, the initial aldol condensation with the sugar is followed by cleavage of water and cyclization to yield a furancarboxylic acid derivative,



Cannizzaro Reaction

The dialdehydes obtained by periodate oxidation of polysaccharides and of glycosides have been found to undergo intramolecular Cannizzaro reactions very readily (85). For instance:



These reactions seem analogous to the conversion of polymeric glyoxal to glycolic acid which takes place in the presence of deuterium oxide without deuterium exchange (86). Although a mechanism involving rearrangement of an intermediate hemiacetal by hydrogen migration has been suggested to account for the lack of deuterium exchange in this reaction, it does not account for the catalytic effect of peroxides (87) which has been observed for other Cannizzaro reactions.

Catalysis by peroxides requires a free radical mechanism, and recently an argument has been presented to support such a mechanism for the Cannizzaro reaction in general (88). The essential reaction sequence is based on the occurrence of the radicals $R\dot{C}O$ and $R{-}\dot{C}H{-}OH$ (formed from the aldehyde through univalent oxidation or reduction, respectively, the dots indicating a free radical form) and may be written as follows:

1. $$R{-}\dot{C}{=}O + HOH \rightarrow R{-}\overset{\displaystyle OH}{\overset{|}{\underset{\displaystyle O}{\overset{\|}{C}}}} + \cdot H$$

2. $$H\cdot + R{-}CHO \rightarrow R{-}\dot{C}H{-}OH$$

3. $$R{-}\dot{C}H{-}OH + R{-}CHO \rightarrow R{-}CH_2OH + R{-}\dot{C}{=}O$$

This mechanism provides for the transfer of hydrogen from one carbon to another without deuterium exchange and explains the formation of hydrogen gas by the action of weak alkali and metallic catalysts on various aldehydes. In the presence of alkali and nickel, aldoses yield salts of aldonic acids and sugar alcohols through the Cannizzaro reaction, but in the presence of alkali and platinum they give salts of aldonic acids and hydrogen in high yield (89). Whether the hydrogen is liberated as a gas or is used in step two of the reaction sequence depends on the metal catalyst. Presumably, in the presence of platinum the hydrogen atoms are adsorbed and combine to give molecular hydrogen.

The free radical mechanism accounts for the experimental observations and is applicable to most Cannizzaro reactions. The previously mentioned reaction of the dialdehydes, however, seems peculiar in that the product is exclusively of the intramolecular dismutation type, and hence it seems desirable to ascertain whether this specific reaction is catalyzed by peroxides before drawing conclusions as to its mechanism.

Sugars

Monosaccharides.—L-Galactose, although long recognized as a constituent of the polysaccharides of certain plants, has been found for the first time in a material of animal origin, the polysaccharide galactogen, derived from *Helix pomatia* (90). D-Psicose (D-allulose) has been isolated and identified as a nonfermentable constituent of cane molasses (91). Recent work has shown that the commonly accepted structure for digitalose is questionable (92). In 1892, Kiliani suggested a 2-methyl-6-desoxyhexose structure, later shown to have one of four configurations (93), two of which were prepared and eliminated as possibilities (94). The enantiomorph of the third possibility has recently been synthesized and found not to be the enantiomorph of digitalose (92). The only remaining possibility in the 2-methyl-6-desoxyhexose series is 2-methyl-6-desoxy-D-gulose, and since the optical rotation of the closely related sugar, 6-desoxy-D-gulose, differs widely from that of digitalose, the authors question the validity of the assumption that the methyl group is in the 2-position.

Oxidation with lead tetraacetate has been used for the preparation of polyhydroxy dialdehydes (95), sugars containing a second aldehyde group, and for the preparation of a benzylidene derivative of *aldehydo*-L-xylose (96).

Disaccharides.—A disaccharide considered to be 3-(α-D-glucopyranosyl)-D-glucose was prepared by hydrolysis of starch with diastase (97). The phenylosazone of this substance should be identical with the phenylosazone of turanose, 3-(α-D-glucopyranosyl)-D-fructose (98), but the m.p. 160–162° reported by Nakamura (97) differs widely from the m.p. 215–220° of turanose phenylosazone (99). A disaccharide, supposedly 3-(β-D-glucosyl)-D-glucose, was prepared by the enzymatic hydrolysis of laminarin (100). A convenient enzymatic method for the synthesis of gentiobiose has been described (101). Although a 2-(β-D-glucosyl)-D-glucose structure has been proposed for sophorose (102), its properties and the properties of its octaacetate are not in complete agreement with those of 2-(β-D-glucosyl)-D-glucose prepared by the Koenigs-Knorr reaction (103). Other oligosaccharides prepared by the Koenigs-Knorr reaction have been reported (55, 104). The crystalline "difructose anhydride III," which is formed in small quantity during the hydrolysis of inulin, was shown to be 1,2′-2,3′-difructose anhydride (105).

Polyhydroxy Alcohols

Need of glycerol and mannitol for explosives, of sorbitol for the synthesis of ascorbic acid, and of other materials has stimulated investigation of the hydrogenation of carbohydrates. Low-temperature hydrogenation of the monosaccharides and disaccharides gives the corresponding sugar alcohols in nearly theoretical yield (106). Hydrogenation at higher temperatures in the presence of suitable catalysts and other agents causes cleavage of the carbon chain with the production of glycerol and propylene glycol (107). The manufacture of glycerol from sugar by fermentation in the presence of sulfites is discussed (108); the yields of glycerol are reported to be from 10 to 15 per cent of the weight of sugar.

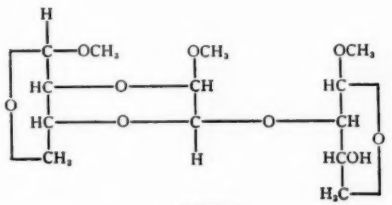
The tetramethyl ether of the naturally occurring anhydro-hexitol, styracitol, has been synthesized by reduction of tetramethyl-D-glucose to tetramethyl-D-sorbitol, followed by formation of a 1,5-anhydro ring (109). The synthesis would seem to establish the 1,5-anhydro-sorbitol structure for styracitol. This work, however, is not in accord with the formation of a derivative of D-fructose from styracitol by indirect oxidation of carbon 5 (110). The latter study supports a 1,5-anhydromannitol structure.

Cyclic Acetals

Benzylidene derivatives.—The possible existence of stereoisomeric benzylidene derivatives has not been sufficiently emphasized until recently when two 1,6-dibenzoyl-2,3,4,5-dibenzylidenedulcitols were prepared (111). These compounds may be a pair of diastereomers differing in the configuration of the acetal carbons of the benzylidene groups or a pair of position isomers, possibly a 2,3-4,5 and a 2,4-3,5 derivative. The large number of isomeric possibilities makes the establishment of structure difficult (96, 112). It is noteworthy that xylose and certain hexoses form *o*-nitrobenzylidene derivatives which on irradiation rearrange to *o*-nitrosobenzoyl derivatives (113). This reaction would appear to provide a means for obtaining carbohydrate derivatives with certain free hydroxyl groups, but attempts to utilize the derivatives for synthetic purposes have not been successful.

Cyclic acetals from periodate-oxidized starch.—Periodate oxidation of starch gives a product which consists of alternate D-erythrose and glyoxal units, connected through the hydroxyls 2 and 3 of the erythrose units and one of the aldehyde groups of the glyoxal units

(114). Treatment of this material with methanol containing hydrogen chloride results in cleavage of some of the erythrose-glyoxal bonds and the formation of acetal derivatives (115). Apparently the hydroxyl of the sugar residue, set free by the cleavage reaction, combines with the free aldehyde group of the neighboring glyoxal unit and forms a cyclic acetal of the type illustrated in the formula (XXXI).



XXXI

The possibility of formation of compounds such as this, either by intramolecular cyclization or by recombination of the initial scission products, and the previously mentioned tendency toward intramolecular oxidation-reduction (Cannizzaro reaction) are factors which should be kept in mind during investigations involving *aldehydo* derivatives.

Oxidation Reactions

Space does not permit consideration of the numerous applications of lead tetraacetate and periodic acid to oxidations of carbohydrates. However, attention is directed to a review of this subject (116) and to the use of periodic acid oxidation in the determination of methyl pentoses (117). Mention might also be made of publications reporting the oxidation of glycals with hydrogen peroxide (118), the oxidation of aldoses with chlorous acid (119), and improved methods for the preparation of various aldonic acids (120) and ketoaldonic acids (121).

The production of calcium 5-ketogluconate and calcium 2-ketogluconate by bacterial oxidation of glucose has been developed, and both compounds are now available at relatively low cost (122). 5-Ketogluconic acid is of particular interest because it may be converted by air oxidation in alkaline solution to tartaric acid (123). Until recently, use of tartaric acid for the production of tetroses and

related compounds has been restricted for lack of suitable reduction methods. A simple process, developed independently in two laboratories (124), consists of conversion of diacetyltartaric anhydride to the acid chloride of monomethyl diacetyltartrate, followed by reduction with hydrogen in the presence of palladium to give methyl diacetyl-L-threuronate. Further reduction with sodium amalgam gives successively L-threonic acid, L-threose, and L-threitol. A cheap source of L-threose would provide raw material for the synthesis of ascorbic acid by the glyoxylic ester method (125).

Errata

We regret that formulae could not be reconstructed in time to permit the following corrections: page 208, Fig. VIII, second formula, insert ( ) next to the upper quaternary nitrogen; page 213, Fig. XXIV, insert H next to the third carbon from the top, counterclockwise.

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THE CHEMISTRY OF THE LIPINS

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Saturated fatty acids.—This field continues to be dominated by the importance of fatty acids with branched chains as components of bacterial lipids. A new representative of this group has been obtained by Peck & Anderson (1) by pyrolysis of phleimycolic acid ($C_{70}H_{138}O_6$), the principal ether-soluble component of the wax of the acid-fast timothy bacillus. The saturated split product is a tetracosanic acid with a branched chain structure (m.p. 75°–76°).

The difficult problems connected with the structural chemistry of these substances has stimulated an increasing amount of synthetic investigations for the purpose of preparing fatty acids with branched chains in the laboratory and of comparing them with the natural products in regard to their chemical and physiological properties. Schneider & Spielman (2) have synthesized the series of methylated acids with even-numbered fundamental chains from methylstearic to methylhexacosanoic acids and several 10-methylated acids. Cason (3) has synthesized 17-methyloctadecanoic acid. Aston and co-workers (4) have continued their work on the synthesis of tertiary acids by the action of sodium alcoholates on secondary alkil-α-bromoketones.

An interesting approach to the structure of branched-chain fatty acids has been made by Stenhagen & Ställberg (5). The authors studied monolayers of various substances of this group dealing with phthioic acid and with phthiocerol in their two latest papers. Their results suggest for phthioic acid the structure of a tri-substituted acetic acid with two long different chains and one short chain, for phthiocerol that of a long chain with only short side chains and with polar groups near one end (6).

The general theory of monolayers of long chain substances has been further developed by Alexander (7), who postulates a vertical orientation of the fatty acid chains in condensed monolayers, and by Kane (8), who concludes that in films of fatty acid mixtures the various compounds do not affect each other.

Unsaturated fatty acids.—Brown and his group (9, 10) continued

their important work on the isolation of unsaturated fatty acids by direct, fractional crystallization at low temperatures and studied methyl arachidonate. The substance was obtained with a degree of purity of 95 per cent and was reasonably free of conjugated compounds, as shown by absorption spectra, diene number, and molecular refraction. The average polybromide number of their samples was 91.8.

The authors re-examined the structure of the methyl ester of arachidic acid by oxidative degradation. Ozonolysis yielded caproic, acetic, glutaric, succinic, and malonic acids, acetaldehyde, and carbon dioxide. Oxidation by potassium permanganate in acetone yielded caproic, glutaric, succinic, and oxalic acids. The structure of arachidic acid was assumed as 5,8,11,14-eicosatetrenoic acid in agreement with conclusions reached by Dolby, Nunn & Smedley-MacLean (11). According to this structure, the chain of the terminal ten carbon atoms is identical with that of linoleic acid. The British workers suggest the possibility of considering linoleic acid as the biological precursor of arachidic acid.

A number of unsaturated fatty acids have been synthesized: Lauer, Gensler & Miller (12) obtained 2-pentadecenoic and 2-heptadecenoic acids by means of a method which permits the transformation of higher, saturated fatty acids into unsaturated acids with one more carbon atom. A new conjugated isomer of the linoleic series 10,12-octadecadenoic acid (m.p. 56, 3°–57°) has been prepared by von Mikusch (13) from the isomerization products of dehydrated castor oil by treatment with caustic soda in an aqueous medium (commercial conjugation). Hexadecenoic acid (bisnoroleic acid) (14), *cis*- and *trans*-10-eicosene, 1,20-dicarboxylic acid (15), and 2,6-phytadienoic acid (16) have been synthesized.

Oxidation of unsaturated fatty acids.—The kinetics of the autoxidation of oleic acid have been studied by Henderson & Young (17). The authors obtained evidence that the formation of peroxides represents the initial reaction and that the rate of the autoxidation after the induction period is a function of the concentration of peroxide oxygen. Treibs (18) investigated the autoxidation of the glycerides containing oleic, linoleic, and linolenic acids, and compared it with that of the methylesters of the individual acids.

Haurowitz and co-workers (19) found that the autoxidation of linoleic and linolenic acids was catalyzed by hemin or hemoglobin under nearly physiological conditions. This effect was coupled with a

gradual destruction of the pigments and the liberation of iron ions. No porphyrins or bile pigments were formed.

A number of new derivatives of fatty acids have been studied with the purpose of facilitating the separation of mixtures and the identification of the individual compounds. Inoue and co-workers (20) described the separation of fatty acid mixtures by conversion into the hydroxamic acids. Kass, Nichols & Burr (21) studied the properties of *p*-phenylphenacetylesters and of the S-benzylthiuronium salts of some octadecenoic acids but found them not suitable for the characterization of substances of the linoleic and linolenic series.

The absorption analysis of mixtures of fats and mixtures of fatty acids has been studied by Claesson (22) with the aid of the boundary method of Tiselius.

The method permits in some instances the detection of small amounts of a component in a mixture if its retardation of the boundary is sufficiently different from that of other constituents. This is the case for some triglycerides. So far, the method could not be successfully applied to mixtures of fatty acids.

Monoaminomonophosphatides.—The outstanding contribution in this field has been made by Folch (23) with his discovery that serine and inositol are constituents of the brain phosphatide mixture hitherto known as cephalin. He succeeded in obtaining phosphatidylethanolamine in pure form (i.e., practically free from serine and inositol) and found that its properties differed strikingly from those hitherto attributed to it. The pure substance is freely soluble in alcohol and contains no inorganic bases although it was isolated without acid precipitation. (It will be remembered that insolubility in alcohol and contamination with potassium and calcium were considered as characteristic properties of the "cephalin" hitherto studied.) The iodine number of phosphatidylethanolamine from beef brain was found to be seventy-eight.

Phosphatidylserine has been obtained practically free from inositol and with only 10 per cent of contamination with nonserine nitrogen. It is strongly acidic and combines with cations which can be removed from the substance by acid treatment. Its iodine number of thirty-three strikingly differs from that of phosphatidylethanolamine.

Inositol phosphatide is still to be considered as a mixture of phosphatides, from which serine, ethanolamine, and glycerol can be isolated in sizable amounts after hydrolysis. It is acidic in character and less alcohol-soluble than ethanolamine and serine phosphatides.

The separation of the individual fractions is accomplished by fractional precipitation with alcohol of a chloroform solution of crude brain cephalin.

Serine phosphatides occur not only in brain but in other organs as well, according to Chargaff and co-workers (24) who investigated the relative proportions of the nitrogen-containing constituents of phosphatides in various organs by the use of the isotope-dilution method. Only egg-yolk phosphatides were found to be free of serine. The serine fraction of the aminonitrogen in the phosphatides amounted to 35.9 per cent in beef brain, 12.7 per cent in beef liver, and 13.3 per cent in pigs' heart. Furthermore, Chargaff reported observations suggesting the existence of nitrogenous phosphatide constituents of unknown nature.

The serine and ethanolamine phosphatides are biologically related to each other and to the cholinephosphatides (25). Stetten demonstrated by feeding experiments with N^{15} serine that a high percentage of the phosphatide colamine was formed by decarboxylation of serine, and it had been previously shown that in the organism colamine was rapidly methylated to choline.

Lesuk & Anderson (26) isolated a saturated lecithin, dipalmitolecithin, from the ether-insoluble lipid fraction of *cysticercus fasciolaris*. The solubility properties of this phosphatide are quite different from those of ordinary tissue lecithins and correspond with the behavior of synthetic phosphatides with saturated fatty acids. The recent experiences regarding the isolation of phosphatides (cephalin, lecithin, sphingomyelin) certainly suggest the greatest caution in the interpretation of the results obtained by the quantitative microfractionation of tissue lipins on the basis of their solubilities.

Glycerophosphoric acid in phosphatides.—Folch (27) showed that the reversible migration of the phosphoric acid radical between the α and β positions of glycerophosphoric acids, under the influence of acids [Bailly (28)], took place under the conditions of the hydrolysis of phosphatides. For this reason, the examination of glycerophosphoric acid obtained from phosphatides by this method does not permit any conclusions regarding the structure of its radical in the phosphatide molecule. Chargaff (29) proved with the aid of radioactive inorganic phosphate that the Bailly migration represents an intramolecular rearrangement, presumably under intermediary formation of a cyclic diester.

Fatty acids in phosphatides.—Peck & Anderson (30) examined

the nature of the fatty acids obtained by hydrolysis of the phosphatides of cell residues obtained as by-products in the preparation of tuberculin. The mixture contained mainly palmitic, tuberculostearic, and oleic acids, and small amounts of stearic and mycolic acids. No phthioic acid was found.

Synthetic investigations.—Ravazzoni & Fenaroli (31) reported a synthesis of choline glycerophosphate by refluxing silver glycerophosphate and bromocholinepicrate. Bersin and his co-workers (32) accomplished the synthesis of a plasmalogen, containing palmitaldehyde and colamine, from silver plasmalogenate (synthetically prepared) and bromocolamine hydrobromide. Sowden & Fischer (33) developed a procedure for the synthesis of optically active diglycerides from the stereoisomers of acetone glycerols and prepared pure *d*-α,β-distearin and pure *d*-α,β-dipalmitin.

Sphingomyelin.—The chief topics of the chemistry of the sphingomyelins during the past few years have been the structure of sphingosine and the question of the presence of the fatty acid groups in amide linkages exclusively.

Carter and co-workers (34) have applied the periodate reaction of Malaprade (35) to N-benzoyl hydrosphingosine and observed no oxidation under various conditions. Since periodate is a specific oxidizing agent for the grouping –CR(OH)–CR(OH)–, the two hydroxyl groups in sphingosine cannot be vicinal as hitherto assumed. N-benzoyl dihydrosphingosine can easily be converted into a cyclic acetal by the action of benzaldehyde and zinc chloride. This reaction is characteristic of 1,2- or 1,3-glycols. On the basis of these results, the authors assign to sphingosine the structure:

$$(CH_3) \cdot (CH_2)_{12} \cdot CH : CH \cdot CH(OH) \cdot CH(NH_2) \cdot CH_2(OH).$$

The presence in bovine brain and spinal cord of O-tetradecylsphingosine has been demonstrated by Niemann (36).

Carter & Norris (37) found dihydrosphingosine as a component of sphingosine fractions obtained from brain and spinal cord. The same compound has been obtained from the cerebrosides of *cysticercus fasciolaris* by Lesuk & Anderson (26).

Klenk & Rennkamp (38) described a method for the preparation of pure sphingomyelin devised to remove monoaminophosphatides which are always found in sphingomyelin samples prepared according to the usual methods and cannot be removed by exhaustive ether extraction. The method of the authors is based on the destruction of

the monoaminophosphatides by saponification with sodium alcoholate at about 70° to 80° while sphingomyelin itself is resistant under these conditions. Our own experiences agree with those of Klenk & Rennkamp inasmuch as we found that all samples of unsaponified brain sphingomyelin contained glycerol and that a part of the fatty acids was split off under mild alkaline hydrolysis. As an explanation of the latter fact, the possibility of the existence of sphingomyelin esters has been previously suggested by us (39). This hypothesis, however, does not take into account the presence of glycerin in sphingomyelin samples.

Recent observations in our laboratory have convinced us that the monoaminophosphatide in sphingomyelin preparations is not a contamination as Klenk & Rennkamp believe, but that it is a constituent of a stoichiometric compound with sphingomyelin. We succeeded in isolating from unsaponified sphingomyelin a well-crystallized substance, the properties of which (elementary analysis, glycerol content, and molecular weight determined ebulliometrically) can only be interpreted as those of a compound of sphingomyelin and a lecithin (apparently a saturated lecithin) in equimolecular proportions. The molecular weight of sphingomyelin isolated after saponification was in agreement with the structure of a simple diaminophosphatide (40).

Cerebrosides.—Danielson, Hall & Everett (41) isolated from the spleen of a patient with Gaucher's disease a cerebroside which contained a fermentable sugar (probably *d*-glucose) as the carbohydrate constituent.

Lipoproteins.—The association of phospholipids with proteins has recently received increasing attention due to the fact that several protein fractions with important physiological effects were found to contain considerable amounts of lipids. Despite the accumulating evidence for the existence and biological significance of complexes between lipins and proteins, it is still an open question as to what extent they can be considered as well-defined chemical compounds such as the proteins with prosthetic groups. It should be emphasized that the lipid part of all investigated lipoproteins contains a number of different lipins—a difficulty not encountered in the chemistry of most of the other conjugated proteins.

Chargaff (42) has examined the classical lipoprotein, lecithovitellin from egg yolk. The substance, prepared in the usual way with some modifications, contained 18 per cent of phospholipids. The phospholipid fraction contained choline and colamine and differed

from the "free" phosphatides of egg yolk only in its higher iodine number (68.5; 57.3 respectively). Heparin was found to combine with lecithovitellin but did not replace the phosphatides as in the case of the thromboplastic lipoprotein from lungs.

In a series of papers, Chargaff and co-workers (43) reported on the chemistry of the thromboplastic protein fraction which can be obtained from lungs by extraction with a dilute sodium chloride solution and isoelectric precipitation. It was found to contain a mixture of phosphatides which were displaced from the protein on addition of heparin. The lipoprotein behaved like a homogeneous substance in the electric field but developed several boundaries during ultracentrifugation.

The virus of the Rous chicken sarcoma I has been purified by ultracentrifugation and represents a high molecular complex of protein, ribonucleic acid, and various phospholipids (44, 45). Fractions of similar general composition have been obtained by sedimentation of extracts from the chicken embryo (46).

The purified chlorophyll protein compounds of chloroplasts have been found to contain a high percentage of phospholipids (47, 48).

Balls and co-workers (49) have isolated a crystallized protein from petroleum ether extracts of wheat flour. The authors conclude from the method of isolation and from the behavior of the substance during the purification that it exists in the flour as a compound with phosphatides. The protein had a bacteriostatic effect *in vitro* (50) which was inhibited by the presence in the medium of small amounts of lecithin, phosphatidyl serine, or inositolcephaline (51). This inhibitory action of phosphatides suggests the existence of specific affinities between phosphatides and protein which would cause the formation of a lipoprotein without antibacterial properties. A specific inhibitory effect of cephalin on the action of other bacteriostatic protein derivatives, gramicidin and tyrocidine, has been observed by Dubos & Hotchkiss (52).

Analytical methods.—Reviews of the fundamental method of Bloor for the determination of lipids and of its modifications have been given by Boyd (53, 54).

Sinclair & Dolan (55) have studied the solubility in ether of phospholipid precipitates obtained according to Bloor by means of acetone and magnesium chloride. This question is important since the ether extraction of such precipitates has been used for the quantitative fractionation of phospholipids for analytical purposes by Kirk (56, 57).

Sinclair found that the amount and the composition of the ether-insoluble fraction was strongly influenced by the amount of the magnesium chloride used for the precipitation of the phospholipids.

Engel (58) has thoroughly examined the various phases of the Reineckate method for the determination of choline and has introduced improving modifications. His procedure requires amounts of choline between 4 and 8 mg. He has also developed a biological assay method based on the prevention of kidney hemorrhages resulting from choline-deficient diets.

A new colorimetric method for the determination of serine has been described by Boyd & Logan (59). It is based on the quantitative conversion of the amino acid to formaldehyde by periodate oxidation and its colorimetric determination with the aid of Eegriwe's reagent.

Hunter (60) in Bloor's laboratory has investigated the sphingomyelin content of various tissues of the cat, using a micromodification of the procedure of Thannhauser and co-workers (61). His paper contains an excellent review of current methods for the determination of sphingomyelin.

Chargaff (29) applied the isotope-dilution method to the determination of the basic constituents of phospholipids.

Brückner (62) described a new procedure for the microdetermination of cerebrosides based on the color reaction of galactose with orcinol and sulfuric acid. This method is more specific than the reductometric method of Kirk (63).

Feyrter and co-workers (64, 65, 66) reported a histochemical reaction for lipoproteins and carbohydrate-containing lipids based on their chromotropic behavior against thionine. The reaction was negative after alcohol fixation. The authors attributed the reaction to carbohydrate components of the stained material which were liberated by the formol fixation. The authors presented evidence that the chromotrophic substances were not identical with the acetalphosphatides.

Phospholipid-splitting enzymes.—Our knowledge about the enzymes acting on the phospholipids proper is still scarce. From the standpoint of the chemist, it would be very desirable to achieve stepwise enzymatic degradations of the lipins—a method which has been applied so successfully to the structural chemistry of other cell constituents of complex structures such as the nucleic acids.

A very important contribution has recently been made by MacFarlane & Knight (67) who demonstrated the presence of a specific lecithinase in bacillus *chlostridium welchii*. This enzyme decomposed

lecithin into phosphorylcholine and diglyceride and was probably identical with the specific α-toxin which is the lethal, hemolytic, and necrotic substance predominant in type A culture filtrates. The activity of the enzyme was specifically inhibited by *chlostridium welchii* (type A) antitoxic sera. The antilecithinase activity of the antitoxic sera ran parallel with their animal protection titre. The enzyme was activated by calcium ions and inhibited by fluoride, citrate, and phosphate. It was relatively heat-stable but readily inactivated by surface denaturation and by sodium dodecylsulfate.

Sperry (68, 69) has reported a considerable decomposition of phospholipids in brain during sterile autolysis. These observations, which are in good agreement with the result of an investigation conducted independently in our own laboratory (70), are somewhat surprising in view of the very slow turnover in brain reported by the investigators who used isotope methods for their studies of phospholipid metabolism. Unlike the effect of the lecithinase of *chlostridium welchii,* the split products formed during brain autolysis were found to be inorganic phosphate, choline, and possibly other bases. No appreciable formation of organic, acid-soluble, phosphate compounds was observed.

Enzymes acting on split products of lipids: Lipoxidase.—Sumner (71, 72) has studied the specificity and the mechanism of the action of lipoxidase. This enzyme is present in soy beans and oxidizes carotene in the presence of unsaturated fatty acids which are converted to peroxides. The authors presented evidence excluding the plausible assumption that the fatty acid peroxides would react with carotene either directly or by peroxidase action. The enzyme acted only on *cis* fatty acids and was highly specific in regard to the structure of the carbon chain and the position of the double bond. Oleic, linoleic, and linolenic acids were rapidly peroxidized while elaidic and erucic acids remained unaffected.

Karrer & Koenig (73) reported that highly branched fatty acids such as phytanoic, phytenoic, and phytadienoic acids are dehydrogenated under the influence of liver and muscle extracts.

Metabolism of the lipins.—Experiments with deficient diets and isotope methods have continued to be the main tools of the investigators.

The effects of choline-deficient diets and their prevention and cure have been intensely studied during the past year. The work deals mostly with two main problems: (*a*) the connection of the chemical

structure of choline with its various biological functions; and (*b*) the choline content of the organs under the influence of choline-deficient diets.

(*a*) From the wealth of important experimental observations, one would gather the general conclusion that the various biological functions of choline (lipotropic effect, growth promotion in the chick, support of growth in rats on homocystine diets, prevention of kidney hemorrhages, prevention of perosis) involve several chemical mechanisms.

Welch & Landau (74) recently reported that arsenocholine $(CH_3)_3\,As \cdot CH_2 \cdot CH_2(OH)^+$ can substitute choline in the biosynthesis of phosphatides but not in its function as methyl-donor. It was found that the phospholipids of rats on an arsenocholine-containing diet contain arsenic in their choline group. The authors conclude that the intact molecule of choline rather than its methyl groups is responsible for its lipotropic action and possibly for its preventive effect concerning perosis and kidney hemorrhages.

Moyer & du Vigneaud (75) found that transmethylation is a highly specific function in regard to the chemical nature of the methyl-donors. Out of almost sixty tested alkyl compounds, only three—namely, choline (and simple derivatives thereof, such as lecithin and phosphorylcholine), betaine, and dimethylethylhydroxyethylammonium chloride—were able to support growth of young rats on a diet containing homocystine but lacking methionine. They found that certain substances of related structure are capable of replacing choline in some but not in all its biological functions. For example, triethylcholine could prevent the occurrence of fatty liver and of kidney hemorrhages in rats; it did not prevent perosis or promote growth in chicks or support growth of young rats on a homocystine diet.

(*b*) It has been demonstrated (76, 77, 78, 79) that the effect of choline-deficient diets on the total choline content differs from that on the phospholipid content of organs (liver, kidney). The total choline content of such animals remains normal and the synthesis of choline from ethanolamine is unimpaired while the phospholipid and especially the lecithin contents exhibit a sharp drop. Stetten & Grail (79) produced a similar effect as well as the development of fatty livers by the feeding of guanidoacetic acid. Creatine cannot transfer methyl groups and is finally excreted as creatinine. Choline acts as a methyl-donor (80) in this irreversible process (81, 82). For this reason, the authors suggest that the feeding of guanidoacetic acid depletes the available

amount of phospholipid choline and that the accelerated destruction rather than the impaired formation causes the decrease of the phospholipid content of the organs. It remains to be seen if a similar theory can be more generally applied to the consequences of choline-deficient diets.

Griffith & Mulford (83) observed that deficiency of the diet in choline and a labile methyl supply in young rats produced a marked elevation of the nonprotein nitrogen content of the blood coincident with the hemorrhagic degeneration of the kidneys. According to Christensen & Griffith (84), choline-deficient diets produced a rapid involution of the thymus gland.

Lowry and co-workers (85) produced liver cirrhosis in rats by a diet with a low choline and casein content. When cystine was added to the diet, a marked improvement in the gross appearance of the liver was observed. After treatment with choline and casein, regeneration of the liver parenchyma took place.

MacLachlan and co-workers (86) analyzed the phospholipid changes in the liver of the fasting mouse, which represents a suitable experimental animal for such studies because of the rapidity of its fat mobilization. The authors found no change in the amount of the total phospholipids per gram of liver tissue and of the lecithin-cephalin ratio but a drop of 50 per cent in the amount of phospholipids per liver and a change in the ratio between the alpha and beta isomers. The authors discussed the significance of the latter finding in view of the isomerization of glycerophosphates during hydrolysis (*27*).

Thannhauser & Reinstein (87) investigated the phospholipid partition in human cases of fatty infiltration and degeneration of the liver occurring as consequence of various diseases.

Fat phosphorylation in the liver was observed in adrenalectomized rats (88). This fact does not support the view that the adrenal cortex plays an important part in the control of lipid phosphorylation in the body.

MacLachlan (89) has investigated the role of the lung in the fat metabolism of the mouse under conditions of rapid fat mobilization or fat absorption from the intestines. No significant changes in the phospholipid content of the organ were found.

The synthesis of phospholipids by tissue slices and minced tissues was studied with the aid of radioactive phosphorus. It was found that brain and nerve tissues are able to build up phospholipids and that this process is enhanced by the addition of various hexoses (90, 91).

Aerobic conditions were found to be necessary for the phospholipid synthesis in all isolated tissues. The results on brain and nerve tissue, which are at variance with the very slow turnover observed in these organs in experiments with labelled phosphorus on the intact animal, bring out a very important point for the interpretation of the results of isotopic experiments in the intact animal: the possibility of slow permeation of the labelled substance into the cells should always be considered when slow turnovers are observed.

Weinhouse & Brewer (92) studied the phospholipid content and the relative proportion of the phospholipids in various functional stages of the corpus luteum.

Blood phospholipids.—Artom (93) has determined the individual phosphatides in human plasma. The values for choline phosphatides were found to be 122 ± 14 mg. and for the cephalin 30 ± 9.5 mg. for 100 ml. of plasma. The paper also contained a comparative study of various methods used for the fractionation of phospholipid in blood.

Artom & Freeman (94) studied the influence of oil feeding on the postabsorptive phosphatide content of rabbit plasma. Elevation of the lecithin fraction was observed, but the authors do not consider their observation as conclusive in regard to the role of lecithin in the transport of lipids. Entenman & Chaikoff (95) studied the effect of overnutrition on the postabsorptive levels of the blood lipids in the dog. Only a slight tendency toward an increase of the phospholipids was observed, but not uniformly. The cholesterol content was not influenced. Raw pancreas did not produce significant changes in the serum lipids of the normal dog.

The large increase of blood phospholipid produced in the fowl under the influence of estrogens has been further studied. Flock & Bollman (96) investigated the partition of the blood phosphatides after administration of diethylstilbestrol and found no significant change in the lecithin-cephalin ratio.

A striking elevation of the level of the blood phospholipids was observed in the pubescent hen by Chaikoff, Lorenz & Entenman (97). The beginning of the abrupt increase can be related to the growth of the oviduct and occurred when the organ reached a weight of 15 to 18 gm. The rise of the blood lipids was preceded and accompanied by an increase in the lipid content of the liver.

Gibbs & Chaikoff (98) reinvestigated the blood lipids in depancreatized dogs with the purpose of differentiating the influence of insulin deprivation from that of pancreas factors influencing the lipid

metabolism. The authors found that a hyperlipemia is not characteristic for fasting depancreatized dogs. Diabetic hyperlipemia was a regular finding only in dogs that received raw pancreas before and after insulin treatment. In these cases the rise of the level of neutral fats was much more striking than that of the phospholipids. The observations of Chaikoff & Gibbs do not concur with the conception of authors reporting on the mechanism of the development of hyperlipemia and fatty livers in diabetes (99, 100, 101).

Entenman, Chaikoff & Reichert (102) observed a considerable increase in all lipid constituents of the blood after removal of the hypophysis and thyroid gland in dogs while the effects of hypophysectomy alone were slight and irregular. The effect of the removal of both glands was ascribed to the deficiency in the thyroid hormone. Maintenance of the animals on a sufficient caloric diet was necessary for the production of the hyperlipemia under those conditions.

A series of excellent reviews on various topics concerning the chemistry and physiology of lipids appeared in *Chemical Reviews,* volume 29 (1941).

Supplement

Fatty acids in phosphatides.—Hilditch and his co-workers (103) determined the component fatty acids of six vegetable seed phosphatides. They found in all investigated phosphatides small amounts of highly unsaturated acids of the C_{20} to C_{22} series which are not present in seed glycerides. The most abundant fatty acid constituent of plant seed phosphatides was usually linoleic acid. The ratio saturated/unsaturated fatty acids was higher in the phosphatides than in the glycerides.

Cerebrosides.—Klenk & Schumann isolated a *n*-hexacosenic acid (m.p. 45°–45.5°) from the hydrolysate of brain cerebrosides (104). Klenk (105) succeeded in isolating a new class of cerebrosides. These substances contain per molecule 1 molecule of sphingosine (or a sphingosine-like base), 1 molecule of stearic acid, 1 molecule of neuraminic acid, and 3 molecules of galactose. They are found in the gray matter of normal brains and, according to Klenk, are constituents of the nerve cells. The term *gangliosides* has been applied to them by Klenk. They are insoluble in ether, acetone, and ethyl acetate, only slightly soluble in alcohol, but readily soluble in mixtures of chloroform or benzene with alcohol. Gangliosides form clear colloidal solutions with water even in concentrations of 10 per cent, and are mono-

basic acids. They do not reduce Fehling's solution, but give a positive red orcinol test (neuraminic acid). No free amino nitrogen is found. Gangliosides are slightly levorotatory: a 5 per cent solution of gangliosides in pyridine gave $\alpha_D^{20} = -2.79°$.

Klenk and his collaborators carried out further investigations dealing with the nature of the carbohydrate in the cerebrosides which accumulate in the spleen of patients with Gaucher's disease. Klenk & Rennkamp (106) reported another case of Gaucher's disease in which the cerebrosides of the spleen contained glucose as the main carbohydrate constituent. Lieb (107) repeated his analysis of the spleen cerebrosides from a patient with Gaucher's disease and confirmed his earlier observation that the carbohydrate in this case was a non-fermentable sugar, probably *d*-galactose.

Klenk & Rennkamp (108) isolated the ganglioside and cerebroside fractions from normal beef spleen. They described three types of cerebrosides in this organ, namely compounds of the molar composition: (*a*) fatty acid : sphingosine : carbohydrate = 1 : 1 : 1; (*b*) fatty acid : sphingosine : carbohydrate = 1 : 1 : 2; (*c*) fatty acid : sphingosine : carbohydrate : neuraminic acid = 1 : 1 : 3 : 1. In each of the three fractions the ratio galactose : glucose was found to be 3 : 2.

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MINERAL NUTRITION

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No summary of the general field of mineral metabolism has been presented in the *Annual Review* since 1936 (1). Various aspects, however, have been covered in succeeding volumes (2, 3, 4, 5, 6, 7, 8). During this period, many papers on mineral metabolism have also been cited in connection with reviews of other topics. Several pertinent review articles have appeared during the past year in other publications. Underwood (9) has summarized the information on the trace elements. The pathological effects of mineral deficiencies have been covered by Follis (10). The mineral nutrition of dairy cattle has been reviewed by Bohstedt (11), and of poultry, by Schaible (12). Within the limitations of the space allowed, the present article deals with some of the earlier papers not falling within the fields recently covered in the *Annual Review,* as well as giving attention to papers in the general field which have appeared in 1942.

Calcium and Phosphorus

Last year the clinical aspects of calcium and phosphorus metabolism were reviewed by Cohn *et al.* (8), and many other papers dealing with these minerals were included in the review on the biochemistry of the teeth by Armstrong (13).

During the current year, several papers have appeared reporting studies of the influence of various factors on calcium and phosphorus absorption. Jones (14) has made an excellent contribution to the knowledge in this field by a simultaneous study of the effects of a number of substances on the pH of the intestinal tract and on bone calcification in the rat. Booth *et al.* (15) have reported that vitamin D-free fats and synthetic triglycerides exert a beneficial effect on calcification in rats receiving a high-calcium, low-phosphorus diet, but not when this calcium and phosphorus relation is reversed. Further evidence on the inhibiting effect of aluminum sulfate on the availability of phosphorus in the rat has been presented by Street (16). Using the Cori technique, Roberts & Christman (17) could not demonstrate any influence of lactose or its hydrolytic products on the absorption of calcium from the intestine. In balance studies with

infants, Knott *et al.* (18) have reported on the relation of calcium intake, vitamin D, type of milk, lactic acid, and honey to calcium retention in infants. The inclusion of honey in the diet improved the retention compared to that observed with corn sugar.

The relationships of dietary calcium, phosphorus, and nitrogen in metabolism have been studied in pre-school children by Hawks *et al.* (19), and in college women by McKay *et al.* (20). Chen (21) obtained no evidence that the levels of intake of calcium and phosphorus influence nitrogen retention. McCance *et al.* (22) have reported that the addition of 100 to 120 gm. of protein to a basal ration containing 40 to 70 gm. of protein increased the absorption of calcium and magnesium by 10 per cent. The urinary excretion was also increased. In a following paper, Lehmann & Pollak (23) reported a study of the effect of amino acids on phosphate transfer in muscle extract in which it was noted that the phosphates and carbonates of calcium and magnesium are much more soluble in solutions of α-amino acids than in water.

An excellent, brief review of osteomalacia as a nutritional disease in China has been presented by Liu (24). It is concluded that it is both a dietary and a hygienic problem and that the dietary deficiencies are calcium and vitamin D, the critical periods being pregnancy and lactation. In studies of the utilization of calcium in vegetables, Shields & Mitchell (25) found that the supply in beets is almost completely unavailable in the rat, but that the calcium in turnip tops and in broccoli is utilized almost as well as that of dried milk solids. Williams *et al.* (26) have produced evidence that the phosphorus in a hay rich in this element is more available to rats than that of a low-phosphorus hay. Breiter *et al.* (27) have studied the utilization of the calcium of carrots in adult humans with results that suggest a somewhat lower availability than the calcium of milk. Gaunt *et al.* (28) have reported a study of the assimilation of radioactive phosphorus following phosphorus deficiency in rats.

Several studies have been reported on the calcium and phosphorus metabolism and requirements of farm animals. Weber *et al.* (29) have published an extensive bulletin on the calcium nutrition of the fattening calf. Beeson *et al.* (30) have reported on the phosphorus requirements of beef steers. The calcium requirements of growing pigs have been studied by Aubel *et al.* (31). Franklin (32) has described hypocalcemia in lambs and ewes and failure of the permanent teeth of lambs to erupt on cereal diets low in calcium. Addition of calcium

carbonate corrected these troubles. Calcium and phosphorus balances in laying hens (33), and the calcium requirement of poultry for maintenance (34) have been investigated by Tyler & Willcox. Studies of the calcium metabolism in the laying hen by Morgan *et al.* (35), using the slaughter method, have confirmed balance studies showing that at the onset of egg production, calcium is removed from the skeleton despite a liberal intake, and that this loss is restored later. Common (36) has reported an extensive study on the relation of acid-base equilibrium on bone metabolism and shell formation during egg production.

The calcium requirements of man are reviewed in the present volume by Sherman & Lanford (37).

Magnesium

The metabolism of magnesium was last covered in the *Annual Review* by Greenberg (4) in 1939. In the same year a more extensive review of magnesium in nutrition was made by Duckworth (38). The later literature has added considerably to the knowledge of this element.

Duckworth *et al.* (39) have noted that on a diet containing only 6 p.p.m. of magnesium, growth of rats ceased after about 4 days and that none of the animals survived longer than 23 days. This low level of intake resulted in a decreased calcification of the bones as shown by a lower ash content. The magnesium content of the bones was decreased, but the percentage of calcium was increased, while the phosphorus content remained unchanged. About one third of the magnesium of the bones was lost during the first 6 days, but further loss occurred at a much lower rate. In continuing their studies, Duckworth & Godden (40), using low-magnesium diets of varying calcium content, found that rats survived longer and that vasodilatation was delayed and diminished on low-calcium intakes. The labile reserves of the bones appeared to be more easily mobilized on the low-calcium diets. These workers explain the two-phase nature of magnesium deficiency on the basis of an initial, very rapid mobilization of the magnesium reserves of the bones for the use of the soft tissues, and later a cessation of mobilization.

Studies have been made on the effect of excess magnesium sulfate on the vascular system by Haury (41), on electrocardiographic changes by Miller & Van Dellen (42), and upon the central nervous system by Bryant *et al.* (43). The effect of magnesium deficiency on

the vago-insulin and sympathetic-adrenal systems has been studied by Gellhorn & Feldman (44). Greenberg *et al.* (45) have discussed factors, including magnesium, which affect the development of tetany in the rat.

Duncan and associates (46), in studying the plasma magnesium of normal cattle during a year's period, observed uniformly low values during May and June, which then increased until November and thereafter remained constant during the winter. Climatic and other non-dietary factors were stated to be concerned. A depressing effect of the lush spring pasture during May and June would seem worthy of consideration as a possible cause in view of studies of "grass tetany." This trouble in cattle was described in detail by Sjollema in Holland (47). Dryerre (48) reported a similar condition in England. The trouble usually occurs soon after cows are turned out to pasture on young, rank grass in early spring. The early symptoms include twitching of the muscles and nervousness. The animals show unusual alertness and a slight disturbance may cause complete collapse in typical tetany. The mortality rate is high. Blood magnesium values are always low; calcium and phosphorus may be normal although Nolan & Hull (49) found slightly low values. The condition has been described in New Zealand by Marshall (50). While the exact cause is unknown, administration of Epsom salts in early stages will prevent tetany. The incidence is decreased by using dolomite in mineral mixtures or by adding it to silage.

Magnesium deficiency is well known to retard the growth rate. A reduced food intake has been observed, but there is also a marked reduction in the efficiency of food utilization. According to Kleiber *et al.* (51) the fasting catabolism of magnesium-deficient rats is increased to 125 per cent of that of controls, and there is a further wastage of nutrients either through increased calorigenic action of the food or by increased excretion of unoxidized materials. There was a decreased efficiency of protein and energy utilization in the magnesium-deficient rats, amounting to about 18 per cent of the intake.

Relatively little work has been reported on magnesium requirements since the reviews of Duckworth (38) and van Veen (52) were published. Daniels (53) recently observed negative to slightly positive balances when pre-school children ingested 10.4 to 11.4 mg. of magnesium per kilo. With intakes of 11.8 to 14.5 mg. per kilo there was a regular retention of magnesium. Her requirement figure of at least 11.8 mg. per kilo essentially confirms previously recommended

intakes. Almquist (54) reported that chicks appear to need about 400 p.p.m. of magnesium in their diet during the early weeks of life. This amount is much higher than is needed by rats. In continuing their studies with calves, Huffman *et al.* (55) found that a daily intake of 12 to 15 mg. of magnesium per kilo of body weight would maintain normal blood serum magnesium levels when the mineral was supplied by alfalfa hay, corn, or corn gluten. When magnesium oxide was added to whole milk, 30 to 40 mg. were needed to prevent hypomagnesemia. The observation of Day & Orent-Keiles (56) that rats fed a diet of cow's milk do not develop magnesium deficiency suggests that calves have a higher requirement for magnesium than rats.

Potassium

The metabolism of potassium was last treated in the *Annual Review* in 1934 (57). Since that time an excellent discussion of this mineral, with special emphasis on its functions in relation to human physiology, has been published by Shohl (58), and certain aspects have also been reviewed by Myers & Muntwyler (59) and Fenn (60).

A detailed picture of uncomplicated potassium deficiency in rats has been presented by the studies of Orent-Keiles & McCollum (61). When young rats were fed a diet supposedly adequate in all respects but containing only 0.01 per cent of potassium, growth continued but at a slow rate, and the length of life did not seem to be affected. There was a roughness and thinning of the fur, and the animals showed an extreme alertness and a marked pica. Ovulation was irregular and at a slower rate with occasional cessation of estrus. Sexual maturity was delayed. The deficient rats stored 2.5 times more magnesium than the controls. The potassium in the muscles, heart, and kidneys was lowered, while sodium increased. All of the potassium-deficient animals exhibited necrosis of the cardiac musculature and damage to the renal tubular epithelium, but other tissues were normal according to a report by Follis *et al.* (62). Cardiac and renal hypertrophy was observed. These studies confirm earlier experiments with rats by Schrader *et al.* (63) and by Thomas *et al.* (64) that cardiac and renal injuries result from potassium deficiency. They did not confirm the reports of Schrader *et al.* with respect to the occurrence of lesions of the pancreas and gastrointestinal tract, nor the findings of these workers and of Heppel & Schmidt (65) that young rats fed a diet containing 0.015 per cent of potassium died

in four to eight weeks. In potassium-deficient rats, according to Heppel (66), serum potassium falls to about one half normal, and the chloride is about 15 per cent lower. The muscles lose potassium and take up additional sodium, but the liver tissue shows no large changes in potassium content.

Sykes & Alfredson (67) found that potassium deficiency caused a decline in the blood plasma potassium from the normal of about 20 mg. per cent to one half that level in two or three months. This drop was accompanied by alterations in the electrocardiographs. The most pronounced change was an increased QRS interval, which in the cases studied did not return to normal even when blood plasma potassium levels were increased by potassium feeding. Lesions of the Purkinje network were found by Sykes & Moore (68) to be associated with the electrocardiographic changes, but no evidence of myocardial damage was noted. Injury to the cardiac musculature has been reported in hogs suffering from potassium deficiency by Thomas *et al.* (64), but a lack of vitamin B_6 was also thought to be a necessary condition. That thiamin is concerned in the picture in the case of rats is suggested by the recent findings of Follis (69) that the myocardial necrosis seen in potassium deficiency is prevented by producing thiamin deficiency at the same time. In a high percentage of potassium- and thiamin-deficient rats, necrosis of the voluntary muscles was seen. Muscle dystrophy did not occur on either potassium or thiamin deficiency alone.

It is of considerable interest that ingestion of potassium salts is immediately effective in relieving familial periodic paralysis, according to Herrington (70) and Gammon (71). This rare disease, characterized by abrupt attacks of flaccid paralysis without sensory loss or psychic disturbances and from which recovery is complete, has been shown to be inherited in certain cases. Males are affected more frequently than females. Without treatment the paralysis lasts from a few hours to several days. The onset of the paralysis is associated with a fall of serum potassium from the normal value of about 19 mg. to below 12 mg. per 100 ml. serum. Ingestion of chloride or citrate of potassium can relieve the paralysis in a few minutes.

Chicks need at least 0.17 gm. of potassium per 100 gm. of diet for normal growth [Ben Dor (72)]. When as little potassium as 0.13 gm. per 100 gm. diet was fed, the mortality was heavy. Purified diet studies with swine by Hughes & Ittner (73) indicate that when the ration contains 0.15 per cent potassium, the growth requirements

are substantially satisfied, but that 0.08 per cent potassium is not sufficient. Based on an experiment of two years' duration, involving two gestation periods and two 90-day lactations with young cows, Du Toit *et al.* (74) tentatively concluded that 30 gm. per day of potassium was adequate for the secretion of two gallons of milk daily. Heifers were able to improve in weight and condition during a dry period on about 25 gm. potassium daily. Heppel & Schmidt (65) found that pregnancy was possible when the diets of rats contained 0.010 to 0.015 per cent of potassium, but the mothers lost body tissue and the young were eaten at birth. During the lactation period a dietary content of 0.58 per cent of potassium usually resulted in some storage, but negative balances were observed as a rule when the diet contained only 0.140 per cent. Young rats were only about one half as heavy as control rats at three weeks of age when the mothers were fed a diet containing only 0.018 per cent of potassium, but the percentage of the element in their carcasses was not appreciably lower.

Sodium and Chlorine

The relation of sodium and chloride to acid-base metabolism has been recently considered by Shohl (58) and by Sendroy (75). There are several significant papers dealing with the effects of deficiencies of these elements. Ornet-Keiles *et al.* (76) found that retardation of growth, disturbances in reproductive functions, lesions of the eyes, and death resulted when rats were restricted to a diet which contained only 0.002 per cent of sodium. A detailed, histological study by Follis *et al.* (77) failed to reveal any specific tissue changes other than those in the ocular apparatus. On a sodium-deficient diet, rats were in negative sodium balance, but retention of potassium and magnesium increased so that the acid-base balance was not appreciably disturbed [Orent-Keiles & McCollum (78)]. Kahlenberg *et al.* (79) have shown that sodium deprivation did not affect the ability of rats to digest and absorb protein and energy. There was, however, a depressed appetite, increased heat production, and decreased body storage of protein and energy. A similar depression of the utilization of protein and energy has been shown to occur when rats are fed a diet deficient in chloride [Voris & Thacker (80)]. Differing from sodium deficiency, however, the drastic restriction of chloride intake does not appear to cause any pathological tissue changes nor death. Marquis (81) and Greenberg & Cuthbertson (82) reported that rats continued to grow at a reduced rate on a chloride-low diet. The

latter authors showed that the urinary excretion of chloride fell rapidly and that rats receiving only about 1 mg. of chloride daily were still in positive chlorine balance.

Iron

Important contributions in the field of iron metabolism have been made in Whipple's laboratory by the use of radioactive iron. Hahn *et al.* (83) have reported evidence that the amount of iron absorbed from the gut is in some manner regulated by the state of the body's iron reserves. Balfour *et al.* (84) found that pregnant women usually absorb two to ten times more iron than non-pregnant women. Little or no iron was absorbed in certain diseases in which the iron stores were known to be abundant (pernicious anemia, familial icterus, etc.). Miller & Hahn (85) found radioactive iron to be rapidly incorporated in the red cells. Within 24 hours the erythrocytes contained as much radioactive iron as was found after several weeks of feeding. Upon disintegration of the red cells within the body, the liberated iron appeared to be nearly quantitatively re-utilized for the regeneration of hemoglobin [Curz *et al.* (86)]. Pommerenke *et al.* (87) reported that plasma iron appeared to be readily transferred to the fetus.

Tompsett (88) studied the absorption of iron by using tests of *in vitro* diffusibility and confirmed a few cases by slaughter experiments with mice. On the basis of these studies he reported that ferric iron cannot be absorbed but that ferric iron is readily reduced in the presence of certain foodstuffs in the stomach rather than in the intestine. He reported also that high-calcium diets reduced the efficiency of iron absorption. In contrast to earlier studies, Black & Powell (89) claim that in both normal and anemic persons an appreciable absorption of hemoglobin iron occurs. The calculations were based on the assumption that the excess iron in feces, in comparison with a preliminary and a post experimental period, represented the unabsorbed iron.

Some attention has been given to the availability of iron from food sources. Sheets & Ward (90) found the iron in legumes more available than the iron of leafy vegetables. Oldham (91) observed that the iron of meat, dried by heating in an oven, was as effective in regenerating hemoglobin in rats as was ferric chloride, while iron from meat, vacuum-dried at low temperature, was significantly less effective. The iron of cocoa resulted in the regeneration of about

two thirds as much hemoglobin in test rats as did ferric chloride, and iron added to a cocoa-milk diet was completely available (92).

Free & Bing (93) reported that several varieties of wheat contained 2.90 to 4.87 mg. of total iron per 100 gm. Chemical studies indicated the iron was 73 to 88 per cent ionizable, and in studies with rats the iron of two varieties of wheat was found to cause almost as much hemoglobin regeneration as equal amounts of ferric chloride. On the basis of long-time balance studies with adult human subjects, Widdowson & McCance (94) found that iron was well absorbed from a diet in which white flour bread constituted 40 to 50 per cent of the calories. Iron was less efficiently absorbed, however, from similar diets when the white flour was replaced with a flour of 92 per cent extraction.

Pfund & Nutting (95) have reported that there was no loss of total iron of potatoes steamed after paring and of those boiled before paring, but there was a significant decrease in the iron content of potatoes pared before they were boiled or soaked six hours before boiling. Potatoes lost no iron during baking but the iron appeared to be concentrated somewhat in the thick, crusty skins. Total and ionizable iron contents of the potatoes were similar and varied from 0.65 to 0.85 mg. per cent. Modified methods have been proposed for the determination of iron in cereals by Andrews & Felt (96) and in biological materials by Parker & Griffin (97).

The human iron requirements are being considered in the present *Annual Review* by Sherman & Lanford (37).

Copper

The evidence concerning the importance of a dietary supply of copper in the utilization of iron for hemoglobin production, first demonstrated by Hart & Elvehjem (1), has been reviewed by Elvehjem (98) and more recently by Schultze (99). Although the exact role of copper in hemoglobin formation is not fully understood, evidence regarding its importance in various enzyme actions continues to accumulate. Schultze (100) has demonstrated that copper is essential for the maintenance of maximum activity of cytochrome oxidase of bone marrow and for its ability to produce hemoglobin. Copper added to a standard diet often causes a moderate increase in hemoglobin production in anemia due to blood loss, but the response is quite irregular in contrast to iron response [Robscheit-Robbins &

Whipple (101)]. They found that the liver and spleen of the dogs contained normal copper stores, indicating that an actual copper deficiency did not exist and suggesting indirectly that the dietary copper had an effect upon enzyme complexes related to hemoglobin production. It has been conclusively shown by Free (102) and by Henderson *et al.* (103) that the graying of hair which is related to copper deficiency is different from that produced by lack of the organic "anti-gray hair factor," pantothenic acid.

The occurrence of copper deficiency in farm animals grazing certain areas which are unusually low in copper content has been reviewed by Marston (104), Underwood (9), and Maynard (7). Recent studies have contributed important additional information. It is well established that "sway-back" or "enzootic ataxia" in unweaned lambs is caused by a deficiency of copper. The clinical symptoms in acute deficiency of very young lambs are spastic paralysis, particularly of the rear legs, incoordination of movement, blindness in some cases, and death, often by an intercurrent infection. In a detailed investigation Innes (105) found a symmetric diffuse demyelination of the white matter of the brain, varying in degree with the severity of the case. In the areas of myelin destruction the axis cylinder had disappeared. A secondary degeneration of the motor tracts was present in the spinal cord. Bennetts & Beck (106) reported that brain lesions are not always seen in the less acute deficiency but lesions of the spinal cord and peripheral nerves are found. In affected lambs the copper content of the blood and liver is lower than normal. Shearer *et al.* (107) have reported that "sway-back" is a simple copper deficiency and that lead is not concerned. Adult sheep suffering from chronic copper deficiency exhibit anemia, diarrhea, loss of body condition, and a characteristic alteration in the qualities of the wool growth (108).

In cattle a chronic copper deficiency occurs in areas where the herbage contains 2 to 4 p.p.m. of copper [Bennetts (109)]. Cattle become unthrifty, fail to gain weight even when grazing lush pasture, and become more or less anemic. The estrus rhythm is disturbed and the animals frequently fail to conceive. Blood copper and hemoglobin values are lower than normal. Copper supplements, fed to the cattle or applied to the grazing land, are very effective in bringing about complete cure, but cobalt, zinc, manganese, and nickel are without beneficial effect. Spontaneous recovery may occur when grazing becomes more favorable. Bennetts and co-workers (110) have reported that

for normal and affected cows, the blood copper was 0.07 to 0.17 and 0.01 to 0.02 mg. per cent, respectively. The livers of normal cows from unaffected areas contained 37 to 231 p.p.m. of copper (dry basis) as compared with 1 to 6 p.p.m. for affected cows. The milk produced was reduced in copper content from normal values of 0.05 to 0.20 mg., to 0.01 to 0.02 mg. per liter for affected areas. Eden (111) has presented data on the copper content of the blood of sheep. Beck (112) has reported that ewes grazing copper-deficient pasture have reduced levels of the element in their milk.

Bennetts and co-workers (113) also report that in very deficient areas, where grasses contain 1 to 2 p.p.m. of copper, cattle showing chronic deficiency may very suddenly die. This trouble is known as "falling disease." Few, if any, gross lesions are seen, but microscopically there is a starvation atrophy of the myocardium with replacement fibrosis, and glomerular lesions of the kidney are found. Death, tentatively ascribed to ventricular fibrillation or heart block, usually occurs soon after calving or during heavy milk production. Horses, sheep, and pigs do not appear to be affected with the acute "falling disease."

Farm animals other than sheep and cattle are not commonly affected in the copper-deficient areas. Bennetts & Beck (106) mentioned, however, that a posterior paralysis of undetermined etiology affecting young pigs shows neurological lesions similar to those seen in lambs. Young foals and calves in the copper-deficient areas sometimes exhibit malnutrition and certain abnormalities in bone structure and in posture, but ataxia is not present.

It is established that the amount of copper in the liver varies with the dietary intake. In a study of copper storage in the liver of sheep made by Edgar (114), a daily supplement of 20 mg. of iron, 0.2 mg. of cobalt, or 25 mg. of manganese were each added to 25 mg. of copper for different groups. No difference was seen in the amount of copper in the liver with the various supplements, but an important increase occurred over animals without copper treatment.

Eden (115) has studied the metabolism of copper in rabbits. On a daily intake of 0.298 mg. of copper, adult rabbits were in copper equilibrium. Most of the copper was excreted in the feces, and even when 50 mg. were fed daily, the urinary excretion accounted for only 1 per cent of the intake. Rusoff (116) compared the copper content of the organs of a new-born calf from a "salt sick" cow with that of a calf from a normal cow. The total copper content of the "salt

sick" calf was greater, and most organs contained more copper than did the normal animal.

Cobalt

Since the work on cobalt, the latest addition to the list of essential mineral elements, was last reviewed (7), several significant papers have appeared. Following the administration of a radioactive isotope to rats, Copp & Greenberg (117) have studied the accumulation of cobalt in the bones, blood, organs, and tissues, and its elimination. Ninety percent was eliminated within two days. Bowstead *et al.* (118) have reported the experimental production of cobalt deficiency in sheep, involving a decrease in the cobalt content of tissues and the impairment of reproduction and lactation.

The possible role of cobalt in hemoglobin formation has been studied by several investigators. Orten (119) has reported results showing that the hypochromic anemia induced in rats fed a low-protein diet responds to cobalt by an increase in both hemoglobin and erythrocytes. Frost *et al.* (120) found that about one half of a group of dogs made anemic by feeding a milk diet responded submaximally with respect to hemoglobin production when given supplements of iron and copper. When such dogs were given cobalt in addition to iron and copper, normal levels of hemoglobin were obtained. These authors feel that while the specificity of cobalt in furnishing an additional stimulus to hematopoiesis is not beyond question, the effect is probably specific. In another paper, Frost *et al.* (121) reported that dogs rendered anemic by feeding a milk diet and subjected to phlebotomy when fed cobalt for a period of three weeks did not show an initiation of hemoglobin production when subsequently fed iron and copper. In some manner the preliminary feeding of cobalt inhibited the expected stimulation of hematopoiesis due to iron and copper therapy. This cobalt inhibition was overcome by feeding liver or liver extracts. Growing dogs maintained on mineralized milk did not show a stimulation of blood formation when fed levels of cobalt which produced a transient polycythemia in adult dogs maintained on mineralized milk. Robscheit-Robbins & Whipple (101) have reported that dogs made anemic by phlebotomy and fed an adequate diet of salmon bread did not show a stimulation of hemoglobin formation when cobalt was given. The addition of cobalt to iron therapy gave no greater increases in hemoglobin than when iron was given alone; in fact, cobalt in high concentrations appeared to inhibit the production of

hemoglobin brought about by iron therapy. The authors state that their results furnish no support for the claim that cobalt causes a polycythemia. Holden (122) studied the differences brought about in blood pigment when the iron is replaced by cobalt. The spectra of cobalti- and cobalto-protoporphyrins and their compounds with globin and other proteins, cyanide, and imidazole are described.

Manganese

Previous studies, referred to in the review by Jukes & Almquist (123) last year, showing a lack of manganese to be concerned in bone malformation and other troubles in poultry, have stimulated work with other species. In contrast to the findings in chicks by Caskey *et al.* (124) that a shortening of the leg bones is involved in manganese deficiency, Barnes *et al.* (125) were not able to demonstrate any abnormal development of the tibia in the rat on a low-manganese diet. Miller *et al.* (126) observed the development of lameness in pigs on diets containing 0.0011 to 0.0014 per cent of manganese, which was apparently prevented by manganese additions. On the other hand, Johnson (127) reported that pigs made a normal growth on a diet containing only 0.3 p.p.m. of manganese, a level which produced a growth-retardation effect in chicks in 14 days and developed perosis in 24 days. A few cases of lameness occurred among the pigs, however, which were attributed to an infection. The manganese content of the bones and certain organs was markedly reduced on the low-manganese diet. Chornock *et al.* (128) found that high-manganese diets retarded growth and induced rickets in rats, but that levels comparable to those correcting perosis in chicks did not retard growth or affect calcification. In continuing their studies to ascertain the mode of action of manganese in the chick, Coombs, Norris & Heuser (129) have produced evidence of a relationship between the manganese content of the diet and the phosphatase activity of the bones.

A critical study of manganese deficiency in the rat has been published by Boyer *et al.* (130), which reveals a retardation of growth as well as reproductive failure in both the male and female. Their studies demonstrated that manganese is not necessary for the synthesis of ascorbic acid in the rat, contrary to the implications of earlier work of others. Hester (131, 132) has reported that the ascorbic acid content of tomato fruits is increased when manganese is added to soils deficient in this element, and this observation has led to the suggestion that manganese may be concerned in ascorbic acid forma-

tion in plants. In a critically controlled experiment by Lyon & Beeson (133), however, it was found that plants, grown in a solution so low in manganese that the content of the fruits was reduced to one tenth that of the controls, with an even greater reduction in the leaflets, produced fruit containing an ascorbic acid content equal to that of the controls.

Fluorine

Several additional papers relative to the influence of fluorine in delaying dental caries have appeared since Armstrong (13) dealt with this question in his review of the biochemistry of the teeth last year. Murray & Wilson (134) have reported a marked negative association of dental fluorosis (mottled enamel) and caries in a survey of 589 school children in London districts. Further evidence that fluorine delays dental caries in rats has been published by McClure (135) and by McClendon & Foster (136). It is evident, however, that the protection is only partial at a level of 10 p.p.m. in the water supply, 50 p.p.m. proving much more effective. Thus, the level which has any significant effect is several times higher than the minimum level which causes dental fluorosis in man. In endeavoring to apply the findings with rats to man, several investigators have noted that caries in the two species is not entirely comparable.

In order to obtain information as to whether increasing the fluorine content of a water supply very low in the element would favorably affect the incidence of caries, Arnold, Dean & Elvove (137) made a study of 109 school children before and two years after the fluorine content of the water had been increased from 0.1 to 0.7 p.p.m. No decrease in the incidence of caries or in acidophilus counts was noted, but it was pointed out that the teeth had already calcified while the children were receiving the low-fluoride water and that the period of higher fluoride consumption was short.

Various aspects of fluorine in relation to dental health are thoroughly covered in a series of articles by leading investigators in the field, which has been published by the American Association for the Advancement of Science (138). It seems to be agreed that although fluorine has seriously harmful effects on the teeth where a certain low level of intake is exceeded, there may be a still lower level where beneficial effects result, at least under certain conditions. No agreement is reached as to how fluorine exerts its possible beneficial effect on dental caries, whether through structural changes, through contact

in the mouth, or through other means. It is apparent that stepping up the intake of fluorine as a means of combating dental caries is not to be recommended under any conditions at present.

A study of the fluorine content of human blood, revealing a level from 27 to 74 μg. per gm., has been made by Hartmann *et al.* (139). Glock *et al.* (140) have made a study of the fluorine content of human rib bones; this work reveals an increase with age. In studies with rats they found that the increase in the bones resulting from the feeding of sodium fluoride was gradually eliminated when the fluoride was withdrawn from the diet. Hoffman *et al.* (141) have reported a detailed study of the growth and histological changes in the teeth of rats on the different diets containing .05 per cent of sodium fluoride. Lawrenz & Mitchell (142) have found that an increase in the concentration of calcium in the diet depresses the retention of fluorine in the body and its deposition in the teeth and soft tissues. The fluorine content of teeth and bones in fluorine-poisoned animals and man has been studied by Danckwortt (143). Hatfield *et al.* (144) have reported a study with sheep which shows bone injuries at a level of 1.5 mg. per kilo of fluorine daily (fed as rock phosphate) with increasing injury at higher levels. The fluorine problem in farm animals has been reviewed by Mitchell (145). MacIntire *et al.* (146) have reported that no increase in the fluorine content of forage crops is to be expected from the conventional use of fluoride-bearing fertilizers and liming materials.

Zinc

Hove *et al.* (147, 148) have described the physiology of zinc deficiency in the rat. Histological studies of tissues of rats fed a diet extremely low in the element have been reported by Follis *et al.* (149). The effects of a deficiency in the mouse have been studied by Day (150). McCance *et al.* (151) have studied the absorption and excretion of zinc in man. Scoular (152) has reported a study indicating that not less than 0.307 mg. of zinc per kilo should be included in the diet of the pre-school child. The zinc contents of various epidermal tissues are reported by Eggleton (153). Eisenbrand & Sienz (154) have reported on the zinc content of the pancreatic juice, and Eisenbrand & Wegel (155) have studied the nature of the zinc-insulin compounds and the combination of the element with glycine, cystine, cysteine, and glutathione.

Minerals and Ageing

Barnes (156) has reported an extensive stereoroentgenographic study of the deposition of calcium in the heart and kidneys of rats during the last half of life under different dietary regimes, in the ageing studies being carried out by McCay at Cornell University. Under all conditions the incidence of calcification in the heart was an increasing function of the age at death. This calcification was less when the food intake was limited than when it was unrestricted. The medulla was found to be the most common site of calcification in the kidneys.

Bazilevich & Pravdina (157) have reported a study of the mineral content of the bones, blood, and urine of aged persons. In healthy persons 90 to 110 years of age the calcium, magnesium, chloride, and phosphate in the blood varied in normal limits like that of younger and middle-aged persons, as did also daily calcium and phosphorus urinary excretions. These findings were in contrast to the increased calcium and phosphorus blood levels and excretions found in pathological conditions accompanied by a decalcification of the bones. Rissel & Wiedemann (158) have compared the potassium and sodium contents of the organs of ten infants with those of adults 30 to 40 years of age. Sodium content decreases with age while potassium content remains fairly constant until middle life when it tends to accumulate.

McCay (159) has critically reviewed the old and new literature of mineral changes in bones, teeth, blood, and in various organs and tissues with age, with particular reference to quantitative changes which are significant in the ageing process.

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THE CHEMISTRY OF THE HORMONES

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All animal hormones which have been isolated up to the present are either steroids or protein and amino acid derivatives. Progress in the elucidation of the chemistry of the steroid hormones, so startling in its rapid achievements in the past decade, now proceeds more slowly with the ultimate goal all but reached. On the other hand, many inroads have gradually been made into the no-chemist's land of yesterday, our knowledge of protein structure. This general development has been paralleled by advances in the field of protein hormones, beginning with the early crystallization of insulin as one of the first biocatalytically active proteins. A review of some of those phases of the chemistry of protein hormones in which progress has been great therefore appeared indicated,[2] even though this would of necessity limit the discussion of papers on the steroid hormones to a minimum. This was felt to be pardonable in view of the thorough treatment which the steroid hormones have received in these reviews in the preceding years.

Thyroid Hormone

Blood thyroid hormone.—The nature of the hormone secreted by the thyroid is still under investigation. It has been recognized that it is not the free amino acid, thyroxine (1, 2). On the other hand, evidence against the occurrence of thyroglobulin in the blood stream is accumulating. Sensitive immunological tests for this protein using the serum of dogs (3) and humans (4) were negative even under conditions of hyperthyroidism; only following trauma to the thyroid gland did thyroglobulin appear in the circulation (3, 4). That the

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[2] The following subjects have regretfully been omitted: adrenalin, intermedin, antihormones, hormones of the parathyroid, of the gastrointestinal tract (including lipocaic, etc.); hormonal control of the pituitary and of lactation; hormonal content of endocrine glands and blood; assay methods.

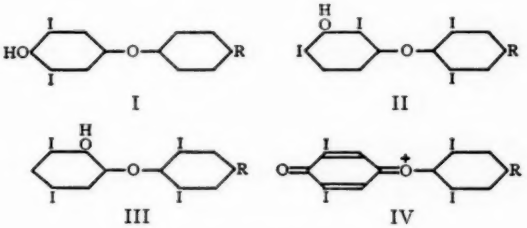
circulating thyroid hormone is not a globulin was demonstrated by blood iodine fractionations in which a parallelism was observed between thyroid function and the iodine bound to the serum albumin fraction (5, 6, 7).[3] This iodine was found in amounts of 2 μg. per 100 ml. of blood in cases of hypothyroidism, of 2.4 to 8 μg. per cent in normal persons, and of 8 to 25 μg. per cent in hyperthyroid cases. No separation of the "hormone iodine" from the serum albumins could be achieved by salting-out methods, dialysis, ultrafiltration, or ultracentrifugation (7). A theory correlating the blood and thyroid iodine findings has been advanced on the basis of the discovery of proteolytic activity in the thyroid colloid (8); it was suggested that thyroglobulin is secreted into the follicles where it is broken down to the smaller protein molecules (such as albumin) which enter the blood stream.

Thyroglobulin.—In contrast to the circulating thyroid hormone, the storage form of this hormone, thyroglobulin, is a readily available protein which can easily be prepared in pure form from thyroid extracts. Studies of the stability of this protein by means of ultracentrifugal analysis indicated its tendency to dissociate and associate, dependent upon protein concentration and other factors (10, 11). The theoretical implications of these findings, as well as of those concerning the primary denaturing action of papain on thyroglobulin, were discussed (12). Part of the amino acid composition was determined by Brand, Kassell & Heidelberger (13). Thyroglobulin with variable and very low thyroxine content (as low as one fiftieth of that found by Brand *et al.*) was described (14); no evidence for the homogeneity of these preparations was given; their polydisperse behavior in the ultracentrifuge was explained on the basis of Lundgren's findings (10, 11, 12).

Thyroxine.—Several new analogues and isomers of thyroxine were prepared using various modifications of Harington's method for the synthesis of thyroxine. The diiodo compound, 3′,5′-diiodothyronine (I), was found less than one fourth as active as thyroxine (15). Of two synthetic isomers (16, 17) differing from thyroxine only in the order of the substituents on the phenolic ring, the compound carrying the phenol group in meta position to the ether oxygen (II) was found inactive, the ortho isomer (III) showed some activity (18). These

[3] Recently the hormonal iodine was found associated with an acetone- and water-insoluble fraction of the serum; it appears probable that the albumins were denatured under the conditions of these experiments (9).

findings support the hypothesis advanced by Niemann, that for thyroxine-like activity a potential quinoid structure (IV) is necessary (16, 17).



I II III IV

(R = $CH_2 \cdot CHNH_2 \cdot COOH$)

In the formation of thyroxine from diiodotyrosine, pyruvic acid and ammonia were found to be by-products (19). Evidence was adduced that the hypoiodite formed acted as the oxidizing agent; a mechanism for this reaction involving a quinoid intermediate was proposed. The effect of progressive iodination on the thyroid activity of serum albumin (20) and casein (21) was studied and the results correlated with the tyrosine (and histidine) content of these proteins.

The site of formation of diiodotyrosine and thyroxine was investigated with the use of radioactive iodine. Chaikoff and co-workers demonstrated a rapid formation of these compounds *in vivo* (22) as well as *in vitro* by intact but not by homogenized thyroid tissue (23). On the other hand, Mann *et al.* (24) believe they have shown that the level of radioactive iodine in the inorganic thyroid-iodine fraction was at no time high enough to be the major source for the iodine of diiodotyrosine. They concluded that iodination of tyrosine takes place outside the thyroid cell, possibly at the level of the cell membrane, an interpretation which may not be regarded as in conflict with Chaikoff's findings (23). Synthesis of thyroxine by tissues other than the thyroid is suggested as an explanation of the observation that the addition of small amounts of iodine to a low iodine diet appeared to benefit thyroidectomized rats (25). Confirmation of these findings, possibly including more detailed data, may be anticipated with interest. Extrathyroid thyroxine synthesis has recently been conclusively demonstrated through isolation of radioactive thyroxine following the administration of radioactive iodine to thyroidectomized rats (26).

Insulin

Chemical properties.—Our knowledge of the chemistry of insulin has not advanced greatly in the past few years. Reviews and viewpoints of 1938 (27, 28) are still correct and intrinsically up to date. To the component amino acids listed at that time the hydroxy amino acids, serine (3.57 per cent) and threonine (2.66 per cent), have now been added (29).[4] The approximate correctness of previous determinations of the basic amino acids was borne out by analyses of insulin-metaphosphoric acid precipitates (30). The ratio of peptide to total nitrogen was determined to be 0.756 (31); when this ratio was calculated from the known amino acid composition (28), the similar value of 0.749 was obtained. To derive from this nitrogen ratio the number of peptide bonds per molecule, an exact knowledge of the molecular weight is essential. On the basis of mol. wt. = 35,700, which in 1939 appeared the most likely value for thoroughly dried insulin, the number of peptide bonds per molecule would be 292 ± 10, a value which is in good agreement with that of 288 postulated by Bergmann. Approximately 90 per cent of the constituent amino acids have now been accounted for as cystine, tyrosine, glutamic acid, leucine, arginine, histidine, lysine, serine, and threonine; the presence of phenylalanine and proline has been demonstrated by isolation (28). All this, to quote Linderstrøm-Lang (31), "gives again the impression that no surprising secret is buried in the purely chemical composition of insulin." While these studies suggest that each molecule consists of only one peptide chain, a molecular structure with eighteen short chains was recently postulated on the basis of amino-nitrogen determinations (32). The evaluation of this finding has to be postponed until more detailed data become available.

The zinc content of insulin crystallized in various ways was studied with radioactive zinc (33, 34). It was found to range from 0.3 to 0.6 per cent, dependent upon the pH of crystallization and independent of the amount of excess zinc in solution; repeated extractions with water caused the zinc in all fractions to decrease to about 0.3 per cent. Crystalline insulin of an even lower zinc content (0.153 per cent) was prepared by Sahyun (35) without addition of zinc, directly

[4] According to a private communication by Dr. Nicolet, the ammonia formed in excess of that due to serine and threonine and tentatively attributed to "other hydroxy amino acids" is actually due to the decomposition of part of the cystine during periodic acid treatment.

from pancreatic extracts. The large scale preparation of crystalline insulin as used at the Connaught Laboratories was described in detail (36). Comparison of crystalline zinc insulins prepared from two new sources (human and bison pancreas) indicated that they did not differ from beef, pork, lamb, or fish insulins in potency and crystalline appearance (37). The identity of insulin derived from these various species was also demonstrated to be immunologically the same (38).

Physicochemical properties.—The solubility of insulin in various solvents and its dielectric properties were investigated by Cohn *et al.* (33, 34). Electrophoretic studies illustrated the well-known limitations of this method; thus, preparations of insulin with lesser potencies than the pure hormone appeared as homogeneous as the fully active crystalline samples (39). Owing to the wide range of insolubility (pH 4.5 to 7.0), the isoelectric point could only be roughly estimated (pH 5.3 to 5.9). Recent redeterminations of ultracentrifugal sedimentation and diffusion constants of carefully recrystallized insulin yielded values of $S_{20} = 3.55 \times 10^{-13}$ cm. per sec. per unit centrifugal field and $D_{20} = 7.53 \times 10^{-7}$ sq. cm. per sec., respectively (40). The molecular weight estimated from these data was 46,000, which is about 30 per cent higher than was previously believed (28, 31). Flow birefringence was observed after heat treatment of insulin indicating the formation of elongated molecules similar to those from other proteins upon denaturation (41). The near-infrared absorption spectrum of insulin did not differ from that of other proteins (42).

Chemical modifications and derivatives.—Freudenberg (43) again affirmed his ability to provoke some reactivation of cysteine-reduced and partly inactivated insulin by the addition of hydrogen peroxide. An ultracentrifugal study of reduced insulin has yielded surprising results (44). Aggregates of high molecular weight were found primarily with only a small part of the protein broken down into the expected low molecular weight fractions. These changes were found to occur at quite low concentrations of reducing agent, paralleling the decrease in biological activity and preceding the appearance of many thiol groups; however, it was recognized that the change in physical state was dependent on the reduction of one or a few disulfide groups. Similar reduction studies in detergent[5]-containing insulin solutions appear of great theoretical and practical interest. The concept that the biological activity of the hormone is dependent upon the molecular size and shape of the insulin molecule rather than on

[5] Duponol.

the existence of any one or several disulfide groups is supported by these findings.[6] This is also indicated by the lack of biological activity of plasteins obtained by the action of trypsin and papain on digests of insulin (46). The inactivity of ultrafiltrates of the partially digested and inactivated hormone also points in this direction (47). Previous results of experiments with ketene on the essential nature of free tyrosine groups (28) have been confirmed (43); this process also has been claimed to be to some extent reversible through careful alkaline hydrolysis. On the other hand, previously claimed reactivation of alkali-inactivated insulin could not be reproduced by the same author, nor could thiol groups be demonstrated in such insulin. The isolation by du Vigneaud *et al.* (48) of considerable amounts of lanthionine from insulin treated with dilute alkali throws some light on this problem.

Complexes of insulin with piperidine and primary bases were crystallized with and without zinc (49). Loss of activity was observed only when such crystals were dried. Several azo derivatives containing up to fifteen azo groups per molecule (calculated for mol. wt. = 40,000) were prepared by Reiner & Lang (50); two of these compounds were obtained in crystalline form (51). The activity of the hormone was somewhat impaired by positively substituted azo groups and less by those containing negative substituents. Introduction of radioactive iodine into the benzene nucleus before diazotization and coupling enabled the authors to follow the rate of absorption of such an insulin derivative; it was found to disappear from a subcutaneous depot at a rate similar to and shortly preceding the drop in blood sugar (52). The fate of insulin in the body is still unknown. The rapid rate of disappearance of the hormone from the circulation was found decreased after exclusion of the kidney from the circulation (53). Yet recent careful work did not reveal the presence of a detectable amount (0.25 unit per 100 cc.) in the urine of normal persons (54).

Anterior Pituitary and Related Hormones

Great advances have been made in recent years in our knowledge of the hormones of the anterior pituitary. Where not very long ago there was doubt concerning the existence of individual hormones,

[6] The *in vivo* inhibition of insulin hypoglycemia by cysteine was shown not to be due to disulfide reduction but to the adrenaline releasing action of the amino acid (45).

there are now several pure or almost pure proteins which have been characterized physicochemically and biologically as thoroughly as insulin and other simpler hormones.

Six active principles have been isolated from anterior pituitary extracts and purified to such an extent that they have now become generally accepted. Most of the biological effects of crude extracts have been shown to be due to these known hormones; others may yet be so identified; others again, but probably not many, will gradually be separated and purified to join the group of the accepted six. The belief that these active fractions might represent artifacts and split products of a hypothetical "macromolecule" has not been supported by evidence. The demonstration of fractionation under the gentlest conditions (55) and the good agreement in the properties of the purified hormones isolated by different groups of investigators militates against this concept unless it be assumed that the macromolecule represents a very loosely bound association product of the individual hormones.

The isolation in pure form of several pituitary and related hormones has revealed a surprising fact, so general in nature that attention will be drawn to it now. It is that of organ and species specificity of these hormones. Thus it was found that the pituitary gonadotrophins were chemically and biologically clearly different from those of chorionic or endometrial origin.[7] The differences in the pituitary hormones of swine, sheep, and beef represent a more recent finding. On the basis of the apparent identical nature of insulin obtained from various species, the same had been assumed also for other protein hormones. Yet minor differences in the make-up of insulin (as they have been observed for beef and sheep lactogenic hormone) have by no means been excluded. On the other hand, pronounced differences in the chorionic gonadotrophins of human and equine origin have been known for some time and might have prepared the minds of investigators for the shock of suddenly finding themselves confronted by an infinitely greater number of hormones than they had expected.

Lactogenic Hormone

Physicochemical properties.—A method of purification of this hormone was described by Lyons (56). A microcrystalline material

[7] It may be hoped that the confusing term "anterior pituitary-like" for the latter hormones may soon disappear from the literature.

was obtained from such purified preparations by White, Catchpole & Long (57) using insulin crystallization methods. Difficulties were encountered by other investigators attempting to repeat this crystallization (58). In the meantime, however, this question has lost much of its urgency; general agreement has been reached that pure amorphous lactogenic hormone differing in no respect from the crystalline material can be prepared by Lyons' method. Physicochemical and biological data concerning the crystalline material which have recently been published (58) were in good agreement with those previously obtained for the amorphous protein (59 to 67). Crystallization was not found to be advantageous as a practical method for the purification of this hormone.

The homogeneity of lactogenic preparations has been demonstrated by a number of methods. Studies of the electrophoretic mobility indicated the presence of a single substance (58, 59, 68) with identical migration rate for the hormone isolated from sheep and beef pituitaries (60, 64). The isoelectric point was uniformly found to be close to pH 5.70, both by these mobility studies and by Abramson's method (58). When the solubility behavior was investigated in several solvents, hormone preparations from either source obeyed phase rule requirements for homogeneous samples (58, 61), but differences between the beef and sheep hormone became evident (61, 64). Studies of beef lactogenic hormone in the ultracentrifuge gave values for sedimentation constant ($S_{20} = 2.65$ or 2.8×10^{-13}) and diffusion constant ($D_{20} = 7.5 \times 10^{-7}$) from which a molecular weight of 32,000 to 35,000 was calculated (58). Osmotic pressure measurements, on the other hand, yielded for both sheep and beef hormones a somewhat lower value of about mol. wt. 26,000 (64), and from recent diffusion (sintered glass membrane) and viscosity measurements a mol. wt. of 22,000 was derived (69). The dissymmetry constants (f/f_o) calculated from these two sets of data are 1.37 and 1.29, respectively (58, 69). The ultraviolet absorption spectrum (70) and the optical rotation (69) were also determined.

The elementary composition of the pure hormone is that of a typical protein with comparatively high sulfur content (1.8 to 2.0 per cent). Of the constituent amino acids only a few have as yet been determined. These are, for beef lactogenic hormone: tyrosine, 5.5 and 5.7 per cent; tryptophane, 1.3 per cent[8] (58, 62); cystine, 3.0,

[8] From this value a minimal mol. wt. of 15,700 can be calculated. This is in agreement with the observed value of 32,000 but incompatible with a mol. wt.

3.4, and 3.1 per cent (58, 66, 71); methionine, 4.3 per cent; and arginine, 8.3 per cent (64, 71). These values were the same for the hormone from sheep pituitaries with the exception of tyrosine of which there was only 4.5 per cent in this hormone (62, 64). Of the methods of purification of the lactogenic hormone, two seemed to have yielded preparations of demonstrated purity (56, 72).

Chemical modifications.—Ketene treatment was found to inactivate the hormone rapidly (73); since this process was paralleled by a decrease in free amino groups, the essentiality of these groups for the activity was postulated. The rapid inactivation of the hormone by treatment with nitrous acid (74) and phenylisocyanate (75) was regarded as confirmation of this finding. While all these experiments were performed under conditions which were believed to exclude the interaction of other than the amino groups with the reagent employed, a demonstration of the intactness of other groups, in particular the phenolic, would have made the case somewhat more convincing. The essential nature of this latter group was later demonstrated through careful treatment with iodine (63, 76). With progressive iodination of the tyrosine groups the activity was found decreased. The rate of this reaction at various pH levels and the effect of iodination on the isoelectric point of the protein were also studied.

To evaluate the role of the disulfide bonds in regard to hormonal activity the effect of thiols on the protein was investigated (65, 66). A great part of these groups could be reduced to sulfhydryl without loss of activity. However, the reduced form of the hormone was considerably less soluble than the native protein, and thus it was explained that treatment with cysteine removed lactogenic together with other contaminating hormones from growth hormone extracts (77, 78). A surprising difference in the reactivity of cysteine and thioglycolic acid became evident during these studies; the latter caused considerably more reduction than the former and thus led more readily to "true inactivation," i.e., loss of activity through reduction and not through decreased solubility. The existence of reducible groups other than the disulfide in the protein and the specificity of various "thiol reagents" were discussed (66).

Effects of the pure hormone.—Isolation of the hormone in pure

of 22,000 to 26,000. A higher tryptophane value later obtained for unhydrolyzed protein (i.e. 2.5 per cent) was preferred by the authors (64); White, on the other hand, believes the lower value obtained after complete digestion to be more trustworthy (58).

form has led to the identification of several effects of supposedly existing principles as actually being due to, or favored by, this hormone. Some of these are the luteotrophic (79, 80) and "pancreatrophic" (81) action of pituitary extracts, also the water drive factor in *Triturus viridescens* (82) and the stimulation of maternal behavior in mammals (83). Much work has been done recently on the endocrine control of mammary development and lactation; the important role of the lactogenic hormone in this process has been established beyond doubt (67, 84, 85). The isolation from the adrenals of a factor resembling lactogenic hormone, cortilactin (86), was not confirmed (87).

Thyrotrophic Hormone

The isolation from beef pituitaries of a protein of high thyrotrophic activity which behaved like a homogeneous substance in the electrophoresis cell and in the ultracentrifuge was briefly announced by Ciereszko & White (88). Other recent methods of fractionation indicated that the thyrotrophic hormone was a pseudoglobulin, slightly more soluble (in salt and water solutions) than one of the gonadotrophins, ICSH (55, 89, 90). A detailed procedure for the preparation of potent hormone fractions was described (91). Previously obtained indications that thyrotrophic hormone was of such small molecular weight that it could be separated from other hormones (gonadotrophins) by ultracentrifugation could not be confirmed by recent work of the same authors (92). This also explains the lack of success in attempts to remove contaminating gonadotrophin from thyrotrophic solutions by ultrafiltration (93).

The effect of thyrotrophic hormone on the thyroid has been reinvestigated in connection with radioactive iodine studies with particular reference to the role of the pituitary in the synthesis of thyroxine (94, 95, 96, 97). The thyroid functions most specifically dependent upon the pituitary hormone were the rate of iodine uptake and the conversion of diiodotyrosine to thyroxine (94, 95). Investigation of the metabolic effects of purified pituitary hormones showed the thyrotrophic factor to cause increases in liver weight (98) and decreases in the blood and tissue nonprotein nitrogen constituents (99).

Adrenocorticotrophic Hormone (ACTH)

The isolation from sheep pituitaries of a pure protein showing high adrenocorticotrophic activity has been announced quite recently

by Li, Simpson & Evans (100). Homogeneity was demonstrated by electrophoretic and solubility methods; and yet more recently by studies in the ultracentrifuge in J. W. Williams' laboratory (101). The isoelectric point was found to be pH 4.7; $S_{20} = 2.1 \times 10^{-13}$; $D_{20} = 10.5 \times 10^{-7}$. Assuming 0.75 for the partial specific volume, a mol. wt. of 20,000 is calculated from these data. The low isoelectric point and the solubility of the protein in the isoelectric range do not conform with expectations based on the properties of less purified preparations (102). However, similar properties were ascribed by Bates and co-workers to their ACTH preparations (103). The claim of the existence of two ACTH's (104, 105), one affecting adrenal weights, the other lipoid distribution, appears of significance in this connection. The former of these factors was claimed to be demonstrable in the serum of pregnant mares; it could be differentiated from the gonadotrophin by its heat stability. This has also been found by Li (100) to be characteristic of his pure ACTH. Augmentation phenomena produced by combined administration of ACTH with certain other pituitary extracts (106) may possibly be due to this dual nature of ACTH. A method of extraction of this hormone from human urine was described (107).

Biological effects of the hypophysis which have recently been reproduced by administration of ACTH are the anti-insulin or glycotrophic action (108, 109) and increases in liver arginase concentration (110). It has also been recognized that this hormone is an important factor in the diabetogenic action of pituitary extracts.

Growth and Metabolic Hormones

Several methods were recorded yielding growth hormone extracts containing limited amounts of the other hormones (55, 90). Further purification was achieved by two separate means: adsorption on norit followed by elution with phenol (111) and cysteine treatment (78). The latter reaction was used routinely in the most recent procedure for preparation of the hormone (112). The final product showed two components in the Tiselius apparatus. Ultrafiltration experiments of crude extracts (113) indicated for the growth factor a molecular weight resembling and not exceeding that of egg and serum albumin.

Purified growth hormone preparations were almost free from the other five hormones, i.e., they contained no more than 0.5 per cent of each of these (112). Yet various metabolic effects other than growth have been produced with low doses of this hormone. These are nitro-

gen retention (114), pancreatic insulin- and liver-arginase-decreasing (81, 110), myoglycostatic, R.Q. lowering, and other effects (93). Also the specific dynamic action of proteins appears to be dependent upon a pituitary fraction resembling the growth hormone (115). Only through isolation of the hormone in physicochemically pure state can it be decided whether these effects are due to separate factors contaminating growth hormone, whether they are properties of the hormone, or whether the growth activity is due not to one hormone but to a synergistic action of these metabolic factors. Purification of pituitary fractions rich in R.Q. reducing and ketogenic action have been reported by Greaves *et al.* (116); while such fractions were found low in all other hormones, they still contained appreciable growth-stimulating activity, which added weight to the belief that these may be properties of the same substance. On the other hand, a recent comparison of the thermostability of growth and ketogenic activities in crude extracts showed the growth to be more resistant to brief heating than the ketogenic potency; this was interpreted as evidence for the existence of two separate factors (117). Also the properties of liver-fat-increasing fractions from pituitary extracts (118) did not correspond to those of any of the known hormones. Diabetogenic activity has not been successfully separated from the accepted hormones (119). The same is true for other metabolic factors so that, at present, there is little chemical evidence (none of it confirmed) for the existence of a multitude of separate metabolic hormones.

Gonadotrophic Hormones

Physicochemical properties of the pituitary interstitial-cell-stimulating or luteinizing hormones (ICSH).—The isolation in apparently pure form of one of the gonadotrophins (ICSH) from swine and sheep pituitaries was announced simultaneously by two laboratories (120, 121, 122, 123). Comparison of the physicochemical data indicated striking differences between the two products (124). Recent immunological studies have confirmed the nonidentity of ICSH from various species and in particular from swine and sheep (125). On the other hand, the hormonal properties were found to be qualitatively similar although the sheep hormone is somewhat more potent than that from swine (126).

The method of preparation of the swine hormone was described in detail by Chow and co-workers (124). The final product appeared homogeneous in the Tiselius apparatus and in the ultracentrifuge.

The isoelectric point was pH 7.45 and the sedimentation constant $S°_{20} = 6.8 \times 10^{-13}$; from this value and an approximation of $D_{20} = 5.9 \times 10^{-7}$ the mol. wt. was tentatively estimated as 100,000. In contradistinction to these values, sheep ICSH as described by Li *et al.* (122, 123, 127), showed an isoelectric point of pH 4.6 and a sedimentation constant of 3.6×10^{-13} (experiments of Dr. M. A. Lauffer, yet to be reported in detail). Its homogeneity was also demonstrated by solubility studies. Osmotic pressure measurements indicated a mol. wt. of 40,000. The low isoelectric point of the sheep hormone was approximately confirmed by the Squibb-Rockefeller group using a highly purified preparation obtained from Jensen (128), i.e., prepared by a method differing from that of the Berkeley workers.

Chemical analyses indicated 3.8 per cent and 1.0 per cent tryptophane for swine and sheep hormones, respectively (127); 4.5 per cent tyrosine and 5.4 per cent cystine were also reported for the latter hormone. While both hormones, like all gonadotrophins, are glycoproteins, they also differ in their carbohydrate content; sheep hormone contains 4.5 per cent mannose and 5.9 per cent hexosamine (123) and that from swine only 2 per cent (127) or 2.8 per cent mannose and 2.2 per cent hexosamine (129). Other preparations of potencies similar to those of the pure hormone were described by Jensen (128) and Fevold (130).

Chemical properties of the pituitary follicle-stimulating hormone (FSH).—This hormone has been prepared in very active form from sheep pituitaries (128, 131); it was freed from all other hormones with the possible exception of ICSH. Preparations obtained from swine, while considerably less potent, were claimed to be free also from that factor (120, 126). FSH, the only albumin of the pituitary hormones, is more soluble in dilute alcohol, acetone, and in salt solutions than the ICSH; it also exceeds this hormone in its carbohydrate content [10 to 13 per cent carbohydrate and 8 per cent hexosamine in not very potent sheep FSH (132); 4.5 per cent mannose and 4.4 per cent hexosamine in swine FSH (129)].

Physicochemical properties of the chorionic gonadotrophins.—Purification of the human chorionic gonadotrophin (pregnancy urine gonadotrophin, PU) was reported by Gurin and co-workers (133). The most active material (*ca.* 8000 I.U. per mg.) appeared electrophoretically very nearly homogeneous with an isoelectric point of pH 3.2 to 3.3. A careful reinvestigation of its ultracentrifugal behavior also indicated an essentially monodisperse protein of $S_{20} = 4.3 \times 10^{-13}$,

$D_{20} = 4.4 \times 10^{-7}$, the partial specific volume 0.76, and mol. wt. close to 100,000 (134). The total carbohydrate content of PU was about 18 per cent, being composed of galactose (not mannose as in the pituitary gonadotrophins) and hexosamine in the proportions of 2:1 (133). Preparations of similar potency were prepared according to a new method by Katzman *et al.* (135).

Physicochemical studies of a purified preparation of the gonadotrophin from pregnant mare serum (PMS) have yielded comparable results. This material, prepared essentially according to Goss & Cole (136), contained 1000 I.U. per mg. It appeared homogeneous in the Tiselius apparatus over a wide pH range, the isoelectric point being close to pH 2.6 (137). In contradistinction to this, however, a four times more potent sample prepared by Goss showed two components in the electrophoresis apparatus (101). PMS also is a typical glycoprotein, the most active preparations (136) containing about 10 per cent nitrogen, 17.6 per cent galactose, and half as much hexosamine (129). Tyrosine (3.54 per cent) and tryptophane (1.37 per cent) were determined by Li on the less potent material (137).

Chemical modifications of gonadotrophins.—Ketene treatment of sheep pituitaries and chorionic gonadotrophins indicated marked differences in their rate of inactivation. ISCH and PMS showed rapid inactivation (appreciable already after five minutes of treatment), FSH activity disappeared almost as fast, while PU showed only beginning inactivation after thirty minutes of ketenization (138). It was concluded that amino groups are essential for the activity of all gonadotrophins except PU; the latter is only inactivated with progressing acetylation of the tyrosine groups. Experiments with nitrous acid (137, 139) confirmed this difference in regard to PMS and PU while doubt was expressed by Bischoff (139) concerning the interpretation of the finding, apparently on the basis of earlier experiments with formaldehyde (140). Inactivation of PMS by acetic anhydride and phenylisocyanate was regarded as confirming the need of free amino groups for hormone activity (141).

A comparison of the stability of various gonadotrophins to the reducing action of cysteine and thioglycolic acid indicated no differences (142). All gonadotrophins were inactivated by intensive or prolonged treatment while no loss of activity was noted under the milder conditions of cysteine treatment sufficient for the inactivation of insulin. It was tentatively concluded that the integrity of certain unreactive disulfide bonds is essential for gonadotrophic activity. Doubt has been

expressed concerning the interpretation of these findings which were completely confirmed (143). The inactivation of highly active PMS through reducing agents, as well as the presence of disulfide groups in this hormone, were demonstrated by Evans, Nelson & Cartland (144). The well-known instability of human chorionic gonadotrophin in solution was explained as being due to the surprising reducing power of this protein (145). From subsequent work (146) it appeared that this action might be localized in the tyrosine groups since the free amino acid reacted similarly under the experimental conditions. The claim that partial oxidative inactivation could be reversed by addition of hydroquinone was not confirmed by Bischoff (147); however, this difference may be explained by too extensive inactivation in the latter experiments. The fact that PMS could be inactivated by oxidation without destruction of its carbohydrate groups indicates the possibility of a similar reaction (148). This hormone was found to be inactivated by both proteolytic and amylolytic enzymes, which was regarded as evidence that only the intact molecule shows hormonal action (149).

Some effects of the pure (or almost pure) gonadotrophins.—ICSH was found to stimulate (or repair) the interstitial tissue in the gonads of immature or hypophysectomized rats of either sex (123, 126). In birds this leads to striking increases in testis weight (150). In male rats the stimulation of the Leidig tissue causes secretion of androgens and thus a secondary growth of the accessories (121, 123). In females (immature or hypophysectomized), ICSH shows functional effects only synergistically with the FSH. The latter hormone alone was found to stimulate development and growth of ovarian follicles and spermatogenesis, both to a limited extent (126, 131). Swine FSH was claimed to cause no estrogen secretion nor luteinization of follicles at the highest levels tested (120, 126); these phenomena could be produced, however, by administration of both FSH and ICSH, the type of response depending upon the doses used and the period of treatment. Sheep FSH, while of considerably greater potency, was never obtained as free from ICSH as the swine hormone if its ability to cause estrus and luteinization at higher doses is regarded as evidence of contamination with that factor (131). The synergistic effects of the two sheep hormones were found similar to those of the swine hormones (131, 151, 152). The extent of the synergism, as measured by the ovarian weights produced, does not generally approach the effects obtainable with chorionic gonadotrophins. Also in regard to the

stimulation of androgen secretion (as evidenced by the extent of hypertrophy of accessories producible in immature or hypophysectomized male rats) ICSH is far inferior to the chorionic gonadotrophins (153). Yet, fresh pituitaries upon implantation were claimed to be similarly effective as the chorionic hormones while implantation of previously frozen or ground pituitaries was as ineffective as all extracts or purified ICSH (154).[9] On the basis of these experiments the hypothesis was proposed that preparations extracted from pituitary tissue might represent precursors of the actually secreted hormones. It appears significant in this connection that all gonadotrophins isolated from pituitaries, and only these, show the phenomenon of "antagonism" (123, 126, 131, 153, 155).

Comparison of the types of gonadotrophic effect produced by the serum of pregnant mares and by highly purified hormone preparations isolated from this source (PMS) indicated no differences (156) and thus lend additional support to the belief that this is a single hormone (137), notwithstanding the fact that it shows the main effects of both FSH and ICSH. An interesting difference between PMS and all other gonadotrophins is the fact that it apparently remains in the circulation for long periods of time while other gonadotrophins, including horse pituitary extracts, are rapidly destroyed or excreted (93, 157). A molecular weight in excess of those of the other hormones should be expected from this behavior.

Metabolism of Pituitary Hormones

The question as to the ultimate fate of the hormones in the body has received renewed attention. Administered gonadotrophins (both pituitary and PU) were found to disappear rapidly from the circulation; yet urinary excretion accounted for only a small part of the hormone administered (158, 159, 160). The site of inactivation was probably not the liver, spleen, or musculature (159). Seidlin's (161) interesting experiments pointing towards destruction of the trophic hormone by the target gland have been confirmed, at least in regard to thyrotrophic hormone (162). This hormone was found to be inactivated when incubated with thyroid tissue, less so by thymus and lymph, and not at all by a number of other tissues. An opposite con-

[9] The same relationship was found to hold in regard to the ACTH activity of these pituitaries.

clusion was reached on the basis of a different type of experimentation; removal of one thyroid (163) or one ovary (164) was found not to increase the response of the remaining organ to a given dose of thyrotrophic or gonadotrophic hormones. It was concluded that the hormone concentration determined its effectiveness rather than the absolute amount, as might be expected if the hormone were used up by the target gland. In view of the present recognition of organ and species specificity of hormones, conclusions concerning the physiological fate of pituitary hormones (164), reached by administration of human and equine chorionic gonadotrophin to rats, are open to doubt; in the original work of Seidlin's (161) hog ovarian tissue was found most effective in inactivating hog pituitary gonadotrophin while experiments with PMS were "not decisive."

HORMONES OF THE NEUROHYPOPHYSIS

Physicochemical and chemical studies.—A thorough and critical review of the subject was recently presented by Irving & du Vigneaud (165). The question as to the number of hormones responsible for the various effects of posterior pituitary extracts is still under discussion. In contradistinction to the case of the anterior pituitary, there is good experimental evidence available supporting both a monistic and a dualistic point of view. The latter opinion is based on the isolation (and commercial preparation) of two factors, pitressin and oxytocin. Highly purified samples of each of these have been obtained with potencies one-hundred to three-hundred-fifty fold those of the crude powder and with little contamination with the other factor. A recent method of separation is by electrophoresis, either subsequent to preliminary chemical methods of extraction (166) and purification (167, 168) or directly applied to the fresh press juice (169). High potency pressor fractions have been obtained by the former method, showing high sulfur and approximately equivalent cystine content (*ca.* 11 per cent), also much tyrosine (10 per cent). Both hormones were found to be amphoteric with isoelectric points of pH 10.8 for the pressor and pH 8.5 for the oxytocic material (168).

Yet more recently (170) products separated by adsorption on base exchange agents were briefly characterized as of considerably higher potencies and sulfur content; 18.3 and 19.0 per cent cystine and

14.2 and 11.9 per cent tyrosine were found in these oxytocic and pressor preparations, respectively. A striking difference in the arginine content was observed; the oxytocic contained less than 0.8 per cent, the pressor hormone 12.3 per cent of this amino acid. This difference which is in accord with the more basic properties of the latter hormone was suggested as a possible basis for a chemical test. Minimal molecular weights of about 1300 were calculated from these amino acid determinations; this is within the range of molecular weights (600 to 2000) estimated from the physicochemical behavior of less pure preparations (165).

Recent support for the belief that these two active materials may represent breakdown products has come first from ultracentrifugal studies (171, 172). All hormonal activities of fresh press juice were found to sediment at the same rate which corresponded to a mol. wt. of 15,000 to 22,000 (one-third to one-half that of egg albumin). On the other hand, brief heating of such extracts with dilute acetic acid (as it is employed in preparation of the above purified principles) modified the hormonal substances so that they could no longer be concentrated by the ultracentrifugal force employed; the same was the case with the commercial preparations, pitressin, pitocin, and pituitrin. While the possible role of adsorption is hard to evaluate in such experiments, they favor the view that the small molecular hormones do not actually occur in the gland but are formed during the chemical treatment. More recently this concept was supported through the isolation by van Dyke and co-workers (173) of an apparently homogeneous protein of equal pressor, oxytocic, and diuretic activity (about 17 units per mg.). Homogeneity was established by studies of its solubility as well as of its behavior in the ultracentrifuge and in the electrophoresis apparatus. The mol. wt., estimated from $S_{20} = 2.61$ to 2.80×10^{-13}, is approximately 30,000; the isoelectric point, pH 4.8. Possible fallacies in this work have been pointed out by Irving & du Vigneaud (165); it appears that the homogeneity of this protein was established by all three methods only within the narrow range of pH 3.4 to 3.9, which is the same range used throughout the process of isolation. As stated by the above-mentioned authors, "use of the same solvent in the isolation and in the purity tests defeats the purpose of these tests, in that conditions favoring the possible association of adsorbent and adsorbate are thereby preserved." Yet bioassays of various fractions obtained during van Dyke's studies never revealed activity ratios differing from, nor absolute potencies

exceeding, those of the pure protein as might be expected if the small molecular hormones were but adsorbed on a carrier protein. If, on the other hand, it is assumed that the active polypeptides represent parts of the "mother molecule," they must be held by very weak bonds in view of the ease with which they can be released chemically as well as under the conditions of certain electrophoretic experiments (169). If it is assumed that each protein molecule (mol. wt. 30,000) contains one of each of the active polypeptides of mol. wt. 1300 (170), the activities of the latter should be limited to 390 units per mg. The fact that higher activities have been recorded (165, 170) appears to be in disagreement with the mother molecule theory; on the other hand, these may be due to potentiation of the activity during the acetic acid treatment (174).

Investigations of the role of cystine disulfide bonds for the activity of the hormone(s) have yielded surprising results. Sealock & du Vigneaud (175) demonstrated the importance of the disulfide-sulfhydryl system for the activity of the hormonal polypeptides but found the reduced form as active as the disulfide; it appeared that these hormones were more susceptible to inactivation by oxidizing than by reducing agents (165), thus resembling pituitary lactogenic hormone and PU rather than insulin. In view of Bowman's (146) recent work, the possible role of tyrosine in this connection appears worthy of attention since this amino acid was found present in the most active preparations (170).

In contrast to the stability of the active peptides to reduction, van Dyke and co-workers (173) found thiol reagents caused pronounced and rapid inactivation of their protein hormone; as in the case of the lactogenic hormone, thioglycolic acid was more effective than cysteine. The high cystine sulfur of this protein (4.3 per cent) appears significant in this connection.

Effects of purified fractions.—While a chemical separation of pressor and oxytocic activities has been possible, the posterior pituitary factors controlling water balance in mammals have not been separated successfully from these hormones. Thus at present diuretic activity is generally found associated with the oxytocic fraction while pressor extracts act antidiuretically at similar low dosages (4×10^{-7} mg. per kg., dog or rat) (176, 177).[10]

[10] Indications have been obtained that water balance in the frog may not be controlled by the same hormonal mechanism as in mammals (178, 179).

THE RENIN-HYPERTENSIN SYSTEM

Great advances have been made in recent years in our understanding of the humoral mechanism of experimental hypertension. There is good agreement in every respect, except that of nomenclature, between the investigators of North and South America who have simultaneously clarified the endocrine function of the kidney. According to this work [as recently reviewed by Houssay & Braun-Menendez (180)] renin, a kidney protein, acts proteolytically on hypertensinogen,[11] a blood globulin, to produce hypertensin,[11] the actual pressor principle; the latter is gradually inactivated by another blood and tissue component, hypertensinase.[11] Renin thus occupies the interesting position of being both a hormone (i.e., a specific regulator secreted into the blood stream) and an enzyme.

The various methods for the isolation and purification of renin from hog kidneys have recently been critically discussed by Schales (181) who compared these with his own procedure for which he claims simplicity, good yields, and high potency of the final product. Renin appears to be a typical heat-labile globulin, containing no cystine or cysteine (182). The requirement of a blood serum component for the action of renin was recognized at the same time in North and South America (183, 184, 185) with the demonstration of an enzymatic interaction between renin and the blood component (hypertensinogen) by Braun-Menendez and his colleagues (185, 186, 187). The mechanism by which renin is removed from the blood is not yet clear. It was found to appear in the urine only after administration of very high doses (188). Hypertensinogen was characterized as a globulin and seems to have been electrophoretically identified with the α_2-globulin fraction (189); it was found to originate in the liver (190).

Hypertensin, the actual pressor amine, has yielded crystalline salts in the hands of Page & Helmer (191). It is thermostable, dializable, fluorescent; can be precipitated by saturation with ammonium sulfate and by phosphotungstate but not by trichloracetic acid; is soluble in various organic solvents but insoluble in ether; is considerably more resistant to acid than to alkali (185, 191). Hypertensin is believed to be a polypeptide with phenolic groups, essential for its

[11] The nomenclature used by Page and co-workers is: renin activator for hypertensinogen; angiotonin for hypertensin; angiotonin inhibitor for hypertensinase. For the sake of simplicity and clarity, the terminology of Braun-Menendez will be used throughout this review.

activity. This was concluded from the finding that tyrosinase and amino peptidases caused inactivation (192, 193). Inactivation of the tyrosinase by boiling for five minutes prevented also its antihypertensin action; yet forty minutes heating at 60° C. was claimed (194) to destroy the enzymatic but not the hypertensin-inactivating power; doubt was thus cast on the specific nature of this inactivation. The physiological inactivator of hypertensin, hypertensinase, was found to be most active at pH 7.6 to 8.5 (195).

The view that the liberation of hypertensin from its precursor through the action of renin is proteolytic in nature received support when it was shown that pepsin could replace renin in this respect (192, 196, 197, 198). Pepsin was found effective from pH 2 to 6 while the optimum for the action of renin is pH 7.5 to 8.5. Surprising was the observation that the action of pepsin on proteins other than hypertensinogen (casein, egg albumin, etc.) produced some pressor activity; the protein derivatives responsible for this effect resembled hypertensin chemically. The finding that gelatin alone of all proteins investigated yielded no pressor derivatives was regarded as additional evidence for the role of the tyrosine in these active polypeptides (192, 196). In contrast to the nonspecific action of pepsin, renin acts only on hypertensinogen. The species specificity of this system became evident when it was found that renin of certain species (e.g., human) acted only on hypertensinogen of the same species (186, 199).

STEROID HORMONES

The following is intended only as a brief and necessarily incomplete classified list of papers which appeared during the past year. Studies correlating chemical changes with those in hormonal activity will be discussed in preference to purely chemical or biological ones. The reader is referred to a number of recent reviews for more thorough discussion of the subjects: adrenal hormones by Pfiffner (200) and Kendall (201), estrogens and estrogen metabolism by Doisy and co-workers (202, 203).

Chemical Syntheses and Transformations

Natural hormones and derivatives.—The attempts at total synthesis of the estrogens by Bachmann and co-workers are continuing. The preparation and crystallization of a racemic stereoisomer of estrone were described (204); its biological activity was compara-

tively low but the higher activity encountered in the noncrystalline mixture of stereoisomers from which it was isolated made it appear possible that the natural estrone was present in the mixture. An isomer of estrone with a phenolic ring B rather than A was also prepared but found inactive up to 1 mg. (205); an estrone analogue in which the phenolic ring A is joined directly to ring C was obtained in two isomeric forms, one of which was active at 5 mg. doses (206).

Through chemical modification of natural estrogens, homoestradiol (with a six-membered ring D) was obtained and its activity compared with, and found similar to, that of homoestrone (207). The oxidation of estrone by hydrogen peroxide at room temperature revealed the surprising fact that ring D is attacked in preference to the phenolic group (208). With the object of obtaining compounds resembling adrenal steroids, products containing α-ketol or glycerol groups either involving C_{16} and C_{17} (209) or C_{17}, C_{20}, and C_{21} (210, 211, 212), were studied in detail. The preparation of a water-soluble derivative of desoxycorticosterone, the β-glucoside, was described (213, 214).

Synthetic compounds with hormonal activity.—The activity of monoesters and diesters of stilbestrol was studied systematically (215, 216); monoesters were found superior to the more easily prepared diesters; the activity in both series decreased with increasing alkyl chain length. Campbell & Chattaway (217) replaced the *p*-hydroxyphenyl groups in hexestrol with different polynuclear systems and from that and preceding work arrived at the conclusion that "hexestrol is almost certainly the most active member of the most potent series of estrogens yet investigated." Jaeger & Robinson (218) synthesized two ketones of the stilbestrol group ($CH_3{\cdot}CO{\cdot}C_6H_4{\cdot}C(Et)$: $CR{\cdot}C_6H_4OH$; R = Methyl or Ethyl) in the hope that these might show progesterone activity; no such activity was evident but may have been masked by the high estrogenic potency of these compounds. Similar attempts to prepare stilbene derivatives resembling desoxycorticosterone in structure and possibly also in activity showed some measure of success (219). X-ray measurements of the molecular size and shape of stilbestrol and estrone indicated configurational similarity (220). A new synthetic estrogen, D.B.E. (α,α-di-(*p*-ethoxyphenyl)-β-phenylbromoethylene), was characterized by Robson & Schönberg (221) as a moderately active estrogen with very prolonged action upon oral or parenteral administration.

Metabolism and Excretion of Steroid Hormones

The estrogens have been shown to be readily interconvertible in the body. The transformation of α-estradiol to estrone and β-estradiol was again demonstrated (222). Conversely, estrone administration led to excretion of estriol and probably also of α-estradiol by normal men and women (223).[12] A new approach to the problem of estrogen metabolism has been taken by Emmens (224) in comparing the estrogenic activities of a great number of compounds upon systemic and local, i.e., intravaginal, application. This S/L (systemic/local) ratio ranged from fifty to five hundred for the natural and many synthetic estrogens and their esters while being around unity for other compounds. This is taken as evidence that the latter must enter the circulation and undergo modification in some other organ before affecting the vagina while those compounds with high S/L ratios can act directly upon the vagina.

A great proportion of intravenously administered estrone was found to be excreted in the bile (in bile fistula dogs) within three days (225, 226); low recovery from the bile had been reported by others (227) after subcutaneous administration of a low dose of hormone. In contrast to the above findings (226), the view is generally held that the estrogens are rapidly oxidized and inactivated in the liver (228, 229, 230, 231). A comparison of the activity of free estradiol with various of its monoesters and diesters upon intrasplenic injection indicated the protecting action of esterification and thus the probability that the inactivating enzyme attacks the hormone through the hydroxyl groups (230). Various plant enzymes (tyrosinases, etc.) were compared concerning their ability to inactivate the estrogens (232, 233). Biskind (228, 229) showed that the capacity of the liver to inactivate estrogens is dependent upon a sufficient supply of the vitamin B complex.

The inactivation of natural androgens by the liver has also been demonstrated, but methyltestosterone seems more resistant to this process (234). Progesterone has been claimed to be similarly effective when administered subcutaneously or by a route which necessitates its passage through the liver (235); contrary conclusions were reached on the basis of experiments comparing the anesthetic action of high doses of the steroid in hepatectomized and normal

[12] The use of hormones labelled with radioactive or stable isotopes for this kind of study may be looked forward to with interest.

rats (231). Pregnanediol excretion was studied in progesterone-treated rabbits (236) and in chimpanzees during pregnancy (237).

Desoxycorticosterone acetate was found to be inactivated by the liver to a limited extent (238), if at all (239); this was also demonstrated by its effectiveness in low doses upon oral administration (240). Administration of desoxycorticosterone acetate to normal men did not affect their 17-ketosteroid excretion nor did it lead to the appearance of pregnanediol complex in the urine (241); this surprising reduction of a primary alcohol to a methyl group, previously observed by the same authors in one case, has recently been demonstrated to occur also in rabbits (245). Fractionation studies of excreted steroids in normal and pathological individuals are continuing (242, 243). In particular those attempting chemical identification through the use of large amounts of urine appear to hold future promise (244).

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WATER-SOLUBLE VITAMINS

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Plan of Review

With a potential supply of citations which alone would occupy about one half the space allotted for this review, it is obviously necessary to eliminate many highly commendable researches from consideration. This requires that the reviewer establish criteria to serve as a basis of selection.

The studies which he is most anxious to include in the discussion (though he will doubtless fail in many cases) are those which contribute new and crucial information regarding the chemistry or biochemistry of vitamins and those which are most stimulative of further research along these lines. What may be called, for the lack of a better term, "routine research" will have to be pushed into the background though it is admitted that in some cases this type of research is of greatest importance. Research involving predominantly the use of mixtures of unknown composition which contain unrecognized principles will likewise be touched upon lightly, if at all. Clinical records, unless unusually conclusive, and reports regarding poorly defined physiological effects will in general be excluded, with the recognition again, however, that such studies may be extremely valuable. This review does not appear to be the place best suited for the discussion of vitamins from the standpoint of synthetic organic chemistry, and hence such discussions will be abbreviated. The relationship of vitamins to the more practical aspects of human nutrition is treated in another chapter of this volume.

Significance of the Term Vitamin

The previous reviewer on the subject of Water-Soluble Vitamins has raised certain questions regarding the meaning of the term vitamin (1) which merit discussion.

The writer believes that it is not possible to delimit the meaning of the term without taking into consideration the historical fact that at one time the nutritional requirements of animals were thought to be satisfied by minerals, carbohydrates, lipides, and proteins alone. At the same time certain "building stones" entering into the make-up

of fats and proteins (e.g., fat acids and amino acids) were known to be utilized by the animal body in the syntheses of these major tissue constituents. Vitamins, historically, were something new and different.

There has been a resistance to the classification of nutritionally important fat acids (linoleic, linolenic) as vitamins (vitamin F) because they are too closely related to fats which had long been recognized as important food constituents. For the same reason there is resistance to including new amino acids or choline in the vitamin group. Their relationship to food constituents recognized long ago (proteins and phospholipides) is too close. On the other hand, there has never been any resistance, so far as the writer is aware, toward the inclusion of nicotinic acid (niacin) among the vitamins. While it has been known as a compound and even as an occasional constituent of tissues for several decades, it constitutes something new, historically, on the nutritional scene. Inositol and *p*-aminobenzoic acid belong with nicotinic acid among the vitamins because they, too, are not directly related to the classical groups of foodstuffs.

The "B Family" of Vitamins

Studies dealing with distribution (2 to 10) have indicated that the vitamins thiamin, riboflavin, nicotinic acid, pantothenic acid, pyridoxin, biotin, inositol, and folic acid are universally distributed in all living matter. Possibly these are the only known vitamins for which this is true. Published data are not available with regard to *p*-aminobenzoic acid.

Furthermore, there is a tendency for these eight vitamins to be associated together. That is, sources which are rich in one are likely to be relatively rich in the others and poor sources for one are not likely to be rich in others. Cases can easily be cited, however, in which this relationship does not hold; inositol is certainly the worst "outlaw" so far as distribution in animal tissues is concerned (2).

This tendency to be associated in tissues is the basis for the early notion of the existence of a single vitamin B, and supports at present the concept of a "B family" of vitamins. How long this concept will endure is a question.

Aside from apparent universal occurrence and similarities in distribution, the B vitamins have the common ability to stimulate the growth of various microorganisms, thus demonstrating their impor-

tance for lower forms of life. The B vitamins, mentioned above, may be the only known vitamins for which this is true. Information on this point is incomplete.

Still another point in common is the fact that of the various B vitamins which have been studied, all appear to exist in tissues partially in an insoluble, bound form. This is not wholly peculiar to the vitamins comprising the B group, but the phenomenon is prominent among them.

Subdivisions of the "B family."—Already there is evidence to indicate that certain of the B vitamins are more closely related to each other than to the others. In diverse (whole) organisms of the biological kingdom, thiamin, riboflavin, nicotinic acid, and pantothenic acid are associated together to a high degree (11). The other members of the "B family" (if such it is) tend to follow more their individual patterns. Specifically, brewers' yeast is an unusually rich source of the four mentioned above but is an indifferent to poor source of the other four. A similar statement holds, approximately, for various other lower organisms.

When the fundamental, biochemical functioning of the various B vitamins is more fully known, it seems likely that there will appear bases upon which they can be classified into subgroups satisfactorily.

Place of ascorbic acid.—The question may appropriately be raised whether ascorbic acid does not belong fundamentally to the group made up of thiamin, riboflavin, niacin, etc. On the basis of present evidence the answer appears to be negative. While there is evidence for its widespread occurrence, it is partially based upon microchemical tests which are not regarded as conclusive inasmuch as other strong reducing agents may be responsible for positive results. The apparent absence of ascorbic acid from yeast may be taken as an indication that it belongs in a separate category from the other vitamins considered. Ascorbic acid appears not to be specifically stimulative of microorganisms, as are the B vitamins, nor is its existence in a bound form in tissues universally recognized.

A consideration of ascorbic acid in this connection tends to strengthen the idea that the concept of B vitamins has a fundamental and not alone an historical basis.

The size of the "B family."—The number of vitamins which should be included in this group is unknown. Some of the "factors" which have not as yet been identified are mentioned in a later section of this review.

No single species of animal is suitable or sufficient for studying all of the B vitamins, especially if the experiments are carried on only for a limited growth period after weaning. We can hardly claim to have solved the problems of vitamin nutrition of the rat until such a time as we can supply all the vitamins required, beginning at birth and extending through the life cycle. Mice (12) do not maintain a healthy appearance in the presence of all the known vitamins, and guinea pigs (13) appear to have requirements for new vitamins which are not known to be required by rats. One of the advantages of the use of chicks in vitamin studies is the fact that nutritional experiments can be started at an earlier period in their life history. The commonplace observation that their vitamin requirements are more exacting than those of a weaned rat is probably due in a substantial degree to this fact.

As more kinds of animals are used experimentally and as experiments are started at earlier stages of development and continued through longer periods, it is probable that a number of B vitamins not yet identified will come to light.

It also seems probable that in the future nutritional need will not be stressed as much as at present and that tissue constituents will profitably be considered and classified on the basis of their fundamental physiological functioning, regardless of origin. From this basic standpoint ascorbic acid and niacin are just as important to a rat as to a guinea pig or to a human being—only the method of procurement may be different.

Broad significance in biology.—It appears, on the basis of present evidence, that the B vitamins occupy a unique position of importance in the field of biology (11) since they appear to enter into the make-up of organisms from the single-celled protozoa, bacteria, and yeasts, up the scale to the most highly developed forms. Presumably they are constituents of fundamental catalytic systems which are essential to living processes. Also it may be presumed that these catalytic systems have a considerable degree of uniformity in their functioning, regardless of where they are found.

Availability of new tools for vitamin study.—In view of the broad importance which B vitamins appear to have, a whole new field of investigation is developing. Vitamin research involving the use of yeasts and bacteria has been treated by the previous reviewer (1) and elsewhere (14, 15), and will be discussed briefly in the following section. A number of recent investigations of general interest dealing

with the vitamin requirements of bacteria and yeasts may be cited (16, 17, 18). Fungi have also recently been investigated from this standpoint (19, 20). Vitamin research involving protozoa is relatively undeveloped as may be judged by consulting a recent review on nutrition of protozoa (21) and current reports dealing with protozoal vitamin investigations (22, 23, 24). From the standpoint of individual B vitamins and their relationships, the nutrition of invertebrates in general is almost an unexplored field (25) although a considerable number of studies, particularly with regard to insects, have been made. Thiamin, riboflavin, niacin, pantothenic acid, pyridoxin, and biotin are all thought to be growth factors for insects (26). The use of rice moth larvae for the biological assay of thiamin has been suggested (27). Riboflavin is reported to be very abundant in the Malpighian system of the roach (28); the nutrition of the *Drosophila* and the wax moth has been investigated (29, 30); the extraordinary richness of royal jelly in pantothenic acid, first reported by Pearson, has been confirmed, and various other B vitamins have been found to be abundant in the same material (10, 31). Several insects and insect larvae were found to be rich sources of B vitamins (7). Housefly larvae contain an antianemic principle required by trout (32).

Isolated plant roots have been used in several recent studies dealing with individual B vitamins (33, 34, 35).

It seems clear that research on the B vitamins is not restricted to a few animals and that findings of a fundamental nature, which may be of universal application, may be made using any one of the many thousands of organisms in which the B vitamins play an indispensable role.

Microbiological tests.—There are two attitudes toward microbiological vitamin tests, both of which need revision. One group tends to accept the microbiological tests as being "sure fire" and "fool proof," and another regards them as worthless unless checked back against supposedly standardized and meaningful animal tests. In the reviewer's opinion, biological tests for B vitamins in general, whether involving animals or bacteria or yeasts, are subject to substantial errors when unfavorable conditions are imposed, partly because the growth substances and their interplay are not as yet all known. The microbiological tests need to be applied with just as much wisdom as animal tests. They have the tremendous advantage that the number of tests can be very large without an inordinate expenditure of time, and the pitfalls inherent in them can be relatively easily discovered. If animal

tests appear to be more perfect or meaningful, it is in part because of the difficulty involved in proving them otherwise. They simply have not been investigated to the same degree that many microbiological tests have.

An appealing innovation in connection with microbiological vitamin tests has recently been made, involving the use of a single organism (*Lactobacillus casei*) for a number of tests (36). There is no serious doubt about the feasibility of using this organism for all of the tests proposed, except for the pyridoxin test (see p. 331).

General interrelations.—The subject of the functional relationships which exist between the various members of the "B family" of vitamins and between them and other nutritional and environmental factors is one of appalling breadth. If, for example, the requirement of each one may be modified by alteration of the supply of each of the others (which may well be the case), then the subject of requirements is necessarily exceedingly complex. It is probable, in addition, that the supply of other vitamins or nutrients (outside the "B family") and various environmental conditions may influence the demand for a particular member of the "B family." Some of the studies dealing with problems of this kind are cited in the following pages.

The question of imbalance first seriously raised by Morgan (37) has not been cleared up. It seems that fortification of human food with one or more of the B vitamins is certain to improve diets rather than to do damage. Evidence does indicate, however, that one vitamin alone when added to experimental diets of a synthetic character may have a deleterious effect. The finding of Mills (38) that pantothenic acid was toxic when given at the rate of 10 mg. per kg. of food to rats on a synthetic diet tends to corroborate Morgan's finding but is in distinct contrast to the careful pharmacological studies of Unna & Greslin (39), who found that rats could be fed 50 mg. of calcium pantothenate per day (about five hundred times the amount given by Mills) for two generations without any apparent damage or slowing of growth. In the case of these latter animals, the addendum was made to a stock diet rather than to a grossly deficient one.

The complex relationships between the B vitamins, choline, methionine, cystine, etc. in connection with fat metabolism have been reviewed recently by Best (40) and McHenry (41), and space will not permit a discussion of these problems here. Since the 1941 review a number of studies on this subject have been reported (41 to 54). Among the interesting findings is that chicks require choline, for which

methionine is not a substitute (49), whereas rats can apparently reproduce on a choline-free diet (45). The fatty livers of choline-deficient rats are reported poor in vitamin A while the kidneys are extremely rich in this vitamin (47). Brain hemorrhages have been reported in choline-deficient rats (54).

That B vitamins have an effect on appetite has long been recognized. The effect of pantothenic acid on the appetite for salt (55) and the relation of both nicotinic acid and pantothenic acid to electrolyte and water balance have been studied (56, 57, 58). Low sodium chloride intake (59) and low water intake precipitate symptoms similar to a vitamin deficiency (56, 57) so that factors of this sort may have a bearing on vitamin requirements.

Interrelations have been observed between pantothenic acid, pyridoxin, and essential fat acids. Pyridoxin-deficient rats are strongly resistant to the graying induced by pantothenic acid deficiency (60). Results from two laboratories show that linoleic acid, pyridoxin, and pantothenic acid are together concerned in the prevention of dermatitis in rats (61, 62, 63). All three are essential for well-being, but a similar skin condition has in the past been ascribed to a deficiency of each one individually. The effects which each produces are dependent upon the supply of the other factors.

The riboflavin concentration in the liver is found to increase during digestion and assimilation as a result of mobilization from other tissues (64). Pantothenic acid appears to have a direct and specific function in connection with this mobilization. Thiamin depletion tends to diminish the process, but pyridoxin appears not to be an important factor.

Thiamin deficiency causes pronounced disturbance of riboflavin metabolism mainly because of poor absorption of the latter. Riboflavin deficiency does not interfere with thiamin metabolism in the same way (65). Other studies (66) indicate that pantothenic acid plays a role in digestion and absorption; it may, therefore, influence the absorption of other B vitamins.

Urinary excretion of niacin by dogs and riboflavin by both dogs and rats bears an inverse relationship to the level of protein intake, indicating that these vitamins are concerned in protein metabolism (67). Thiamin, riboflavin, pantothenic acid, and pyridoxin appear by the paired feeding method to have similar though not identical effects upon appetite (68), indicating an interrelation of these factors.

That B vitamins are interrelated with other vitamins is indicated

by Russian work, in which thiamin is reported to increase the fixation of ascorbic acid by guinea pig tissues, in this respect functioning the opposite of carotene (69).

Mills (38) has reported an extension of previous work on the effect of temperature on requirements for B vitamins. His work indicates that at 91° (compared with 60°) rats require twice as much thiamin and pyridoxin and over five times as much choline. The pantothenic acid requirement was unaffected by temperature. Chicks required four times as much thiamin and pyridoxin at the higher temperature.

The examples cited are by no means exhaustive but indicate clearly that complex relationships exist between the various B vitamins and other influences. The supply of one B vitamin may affect the actual supply of another through the medium of intestinal synthesis which is discussed in the next section.

Intestinal synthesis.—That B vitamins can be synthesized in the rumen of cattle has been known for many years. This has been studied quantitatively with respect to individual vitamins in recent years (70, 71, 72). There has been renewed interest in intestinal synthesis in rats, and, of course, the possible application of the findings to human nutrition is of importance. Referring to pantothenic acid, Gordon (73) states, "This synthesis probably occurs in most animals, including man; but the site of the bacterial action in the colon makes absorption impossible." The latter part of this statement seems to be more positive than is justified though it seems very likely that intestinal synthesis is relatively unimportant in human beings.

Bacteria in general (six diverse organisms) have been found to synthesize eight B vitamins (8), and biotin production was especially abundant in all except one. Intestinal bacteria were found to produce, with few exceptions, thiamin, riboflavin, niacin, and biotin (74).

Introducing thiamin into the diet of cows increases the synthesis of other B vitamins (70), and pantothenic acid is said to increase intestinal production of other B vitamins in the rat (75). Induction of inositol synthesis in mice by pantothenic acid feeding has been demonstrated (76). This synthesis is thought to be important enough so that what appears to be pantothenic acid deficiency is fundamentally a deficiency of inositol. The fact that inositol deficiency appears to develop even when inositol is in the diet and pantothenic acid is absent may be due to the role of pantothenic acid in aiding absorption (66).

Martin (77) found that a diet containing thiamin, riboflavin, niacin, pantothenic acid, pyridoxin, and choline is just as complete for rats as one containing in addition both inositol and *p*-aminobenzoic acid, and that the addition to such a diet (containing six addenda) of either inositol or *p*-aminobenzoic acid alone precipitated a need for the other. These findings are interpreted in terms of alteration of intestinal synthesis.

The use of sulfaguanidine in purified diets to decrease bacterial action in the intestines has been repeated in a recent study, employing, in addition, succinyl sulfathiazole (78). A number of symptoms have been observed: agranulocytopenia, leukopenia, hypocellularity of bone marrow, and occasionally an anemia; hyalinization, necrosis, and calcification of voluntary muscle; hyaline sclerosis and calcification of blood vessels; also dermatitis, necrosis of heart muscle, liver damage, and hemorrhages. The dermatitis symptoms disappeared when biotin was administered. These drugs are thought to produce their effects either by preventing intestinal synthesis of essential nutrilites or by interfering, themselves, with essential enzyme systems in the body.

That prevention of intestinal synthesis is an important action of such drugs is indicated by the finding that folic acid and biotin are growth-promoting for rats when succinyl sulfathiazole is fed (79). Ordinarily intestinal production of these two vitamins makes difficult a demonstration of their vitamin properties. It is not known how many of the symptoms listed above are connected with induced vitamin deficiencies. The intestinal synthesis of eight B vitamins by rats has been recently studied quantitatively (80). Two diets were fed; each diet was chosen because of its ability to support a different type of intestinal flora. The results indicated that synthesis took place primarily in the cecum, and the extents to which the various vitamins were synthesized and became available were in approximately the order: biotin, pyridoxin, folic acid, pantothenic acid. Relatively small amounts of thiamin, riboflavin, niacin, and inositol appeared to be produced. The character of the diet was found to be an important factor in determining the extent of the syntheses taking place.

It has been found that in young rats the cecum is relatively only 1/30 to 1/50 as large as it becomes in the adult rat (81). Removal of the cecum had no untoward effect on the conditions of rats, provided their diet was fully adequate. However, when cecectomized and normal rats were placed on mildly deficient diets, the normal rats thrived while the cecectomized animals lost weight, became denuded,

and suffered from dermatosis. It is suggested that the contrast in the requirements of old and young rats for B vitamins is due largely to differences in cecal size and the ability to carry on vitamin synthesis. The use of cecectomized rats for studies involving intestinal synthesis of B vitamins seems promising and less liable to produce complications than feeding of bacteriostatic drugs, which may also have other effects.

Relation to metabolism.—We shall mention in this section a few of the studies in which several of the B vitamins have been studied together and have been found to have effects upon metabolism. Studies involving individual vitamins will be treated later under the appropriate headings.

Brief mention has already been made of the problems of the relationship of various B vitamins to fat and protein (68) metabolism. The importance of B vitamins (in the form of brewers' yeast) as compared with the heat stable members of the family (autoclaved yeast) in maintaining the glycogen content of muscles was emphasized in a belated Russian report (82). Deficiencies of thiamin, riboflavin, pantothenic acid, or pyridoxin have been found to result in lowered liver-glycogen levels (83). The administration of any one of the four vitamins to animals deficient in that particular vitamin caused an elevation of the liver-glycogen level. The response was characteristic (over a twenty-four-hour period) for each of the vitamins. While no one vitamin appeared to be peculiarly associated with glycogenesis or glycogenolysis, each appeared to be related to the others and all were necessary for the maintenance of a balanced metabolism.

Riboflavin and pantothenic acid deficiencies were found not to affect the basal metabolic rates in rats, but B_6 deficiency caused an increase in this value (84). The respiratory quotients were found to be increased in all three deficiencies, and incomplete combustion of metabolites was suggested as the cause.

Riboflavin deficiency was found to result in a diminution of succinoxidase activity of the livers of rats, and flavoproteins were concluded to be the components which were in low concentration (85). Biotin and pantothenic acid deficiencies did not cause lowered succinoxidase activity. On the other hand, biotin and pantothenic acid deficiencies did lower pyruvate oxidation (86) in liver and were concluded to be components of the enzyme systems concerned in this oxidation. Studies of this sort are extremely important because of the light that they may throw on the fundamental roles played by the individual B vitamins.

Relation to embryonic development.—Included in studies dealing with distribution of B vitamins was one involving embryonic, young, and mature tissues of the rat and chicken (3). Embryonic tissues were, generally speaking, richer than the tissues of the young. The mature tissues were often richer also, as judged by the contents of the tissue autolysates in all cases. In some cases there appeared to be a large accumulation in mature tissues. The injection of thiamin, riboflavin, niacin, and pantothenic acid individually into eggs before incubation resulted in interesting changes in blood hemoglobin concentration, brain size, and feet size of the chicks after incubation (87). When the injection of the vitamins was accompanied by other untoward influences, such as the use of distilled water as a solvent, the incidence of embryo deformities was increased beyond what it was in the absence of the injected vitamins.

Feeding extra pantothenic acid to hens, which increases its content in the eggs (88), was found to affect the embryos in the same general manner as its injection. The most striking effects were increases (*ca.* 16 per cent) in hemoglobin concentration and decreases (*ca.* 17 per cent) in heart size. There was also a substantial increase in hatchability. Changes of the concentration of various other B vitamins in the chick tissues, as a result of enriching the eggs by feeding pantothenic acid, were also observed, indicating an interrelationship between the B vitamins—a relationship which, however, appeared to be different in the different tissues.

During the incubation of eggs, there is found to be no increase in the riboflavin, pantothenate, or biotin content (89) but a ten- to twentyfold increase in the niacin (90) and about a sixfold increase in inositol content (89). It was presumed that chicks after hatching retain the synthetic powers observed during incubation and that niacin and inositol are probably not required in the diet of the growing chick. Subsequent studies, however, showed that inositol does not actually increase in concentration during incubation (91) but merely changes its mode of combination and that in spite of the synthesis of niacin during incubation, chicks respond to it in the diet (92).

Relation to chemotherapy.—The discoveries of the past few years that the bacteriostatic effect of sulfanilamide can be neutralized in a specific manner by *p*-aminobenzoic acid and that the latter is a naturally occurring vitamin component of tissues may have in them the basis for an entirely new science of chemotherapy. The phenomenal usefulness of sulfanilamide and related drugs is undoubtedly related,

though admittedly in a complex manner, to the existence of *p*-aminobenzoic acid as a fundamental tissue constituent.

Recognition of this relationship suggests the possibility that vitamin after vitamin (particularly of the B group) might serve as a basis for a series of useful chemotherapeutic agents which may act competitively with the respective vitamins. Among the analogues of vitamins which have been studied are pyridine-3-sulfonic acid (93) and pantoyltaurine (94, 95), analogues, respectively, of niacin and pantothenic acid. Analogues of amino acids have also been studied (96, 97). Analogues of pantothenic acid have been thoroughly studied (98, 99), and the *p*-aminobenzoic acid-sulfa drug relations have been studied from several standpoints. Questions of specificity have been debated; complications have arisen which cannot be interpreted on any simple hypothesis (100 to 109). Among the interesting findings is the fact that sulfanilamide itself can function as a growth substance not only for bacteria (110) but for animals (111). A simple hypothesis to explain this fact is that transformation of the "unnatural" aniline derivative to the other (indispensable one) can be accomplished *in vivo*.

While it must be admitted that the new approach to chemotherapy referred to above has not, as yet, led to the discovery of new therapeutic agents of outstanding merit, it seems likely that a more thorough study of the fundamental facts concerned will bear fruit.

Hormone relationships.—The relationships which have been shown to exist between vitamins and hormones are disappointingly few in view of the fact that they have often been associated in the minds of biochemists. A survey of twelve beef endocrine glands for eight B vitamins revealed no examples suggesting a direct connection between any of the vitamins and any one of the hormones or hormone-producing glands (2). (The adrenal glands and the pars intermedia of the pituitary are unusually rich in ascorbic acid, but no direct connection between the vitamin and the corresponding hormone or hormones is known.)

The lack of relationship, in general, was indicated by the fact that in the anterior and posterior portions of the beef pituitary, the vitamin B distribution was identical within experimental error in spite of the vast differences in the physiological functioning of the two glands.

There can be no serious question regarding the indispensability of the B vitamins in the functioning of every gland. Two recent reports seem to show conclusively that the ability of castrate female rats to

inactivate implanted estrone is dependent upon their supply of B vitamins (112, 113). When a pellet of estrone was implanted in the spleen and the animals were fed a good diet, they remained anestrus, but on a diet deficient in B vitamins the animals showed estrus almost continuously. Anestrus in rats has been induced by thiamin or riboflavin deficiency (114).

Somewhat similar in principle is the interesting observation that requirements for various of the B vitamins are increased during thyroid feeding (115). Normally fed rats lost weight when thyroid was fed; this loss could be stopped by thiamin administration, but the rats failed to gain until either a rich source of B vitamins was added to the diet or pyridoxin and pantothenic acid were injected.

Relation to anemias and blood pressure.—It can safely be taken for granted that B vitamins are essential for the processes of hemoglobin and corpuscle building, but whether they have special functions in connection with these processes is not known. A brief report has indicated that the hemoglobin level in women can be raised substantially by administration of thiamin, riboflavin, and niacin and that the same results are not obtained with thiamin alone (116). Pyridoxin or pantothenic acid deficiency has been found to cause anemia in pigeons (117).

The problem of pernicious anemia continues to present difficulties, especially because of the lack of any suitable biological test for the extrinsic factor. A clinical report indicates that three out of four cases of pernicious anemia responded successfully to treatment with "vitamin B complex" preparations, which, however, were reported to be slower in action than liver preparations (118). "Vitamin B complex" preparations which have been on the market are far from balanced in their content of B vitamins (119) so that on this score, alone, too much should not be expected from their use. Trout anemia has already been mentioned (32).

Mild deficiency of heat stable components of the B complex is reported to cause a rise in blood pressure in rats (120).

Relation to cancer.—The first suggestion of a relationship between vitamins and cancer goes back to the early work of Funk. In line with this idea is the recent report that avitaminoses B are probably responsible for precancerous lesions in the mouth and possibly in the stomach and intestines as well (121). One report is that antiblastic constituents of the food are increased by cooking (122); another suggests the name "vitamin N" for the cancer-inhibiting vitamin in foods

(123). Such reports, however, cannot be considered to have a sound scientific basis.

The production of carcinomas in the livers of rats by feeding dimethylaminoazobenzene and the protection against this condition by feeding rich sources of B vitamins have been observed over a period of years in a number of laboratories. No definite agreement has been reached as to the exact agent or agents which are responsible for the protective action though casein and riboflavin together have been found to be definitely protective in their action (124, 125).

The biotin content of malignant neoplasms has been reported to be abnormal (126, 127). In the first report many of the cases covered were those in which tumors had a high biotin content. In studies in the reviewer's laboratory, the biotin content of human and rat cancers was found consistently to be about the lowest, relatively, of any of the B vitamins (6).

The procarcinogenic action of biotin was emphasized in one report in which evidence was presented showing that the addition of biotin to certain diets increased the incidence of dimethylaminoazobenzene-induced hepatomas (128). In the light of accumulated knowledge it appears that the action of biotin, which has been observed to produce fatty livers (129), is probably to impair liver function, thereby to prevent the detoxification of dimethylaminoazobenzene by the liver and thus to affect only indirectly tumor incidence and growth.

Studies dealing with the regressions of spontaneous tumors in mice, resulting from injecting fractions of yeast extracts, have led to the conclusion that no known member of the "B family" of vitamins is responsible for the action (130). Disappearance of the tumors is reported in about 30 per cent of the cases. In another report both pantothenic acid and riboflavin were found to enhance the inhibitive effect of injected yeast extract on tumors (131). In another laboratory supracortin plus members of the "B vitamin family" decreased the number of takes (from implants) to about one-half that in the control rats (132). In another investigation, fortification of stock diet with synthetic B vitamins caused 10 to 15 per cent of the mice to be resistant to cancer implants while the controls were 100 per cent susceptible (133).

That there are some rather direct connections between B vitamins and cancer seems self-evident. An extensive study of the distribution of B vitamins in normal as well as cancer tissues was started in the reviewer's laboratory in 1940 (134) with the intention of helping to

discover these relationships. As a result of these studies a considerable number of cancer tissues from human, rat, and mouse sources were assayed for eight B vitamins (6). Among the conclusions drawn was that cancers from different animals and different sites, regardless of how induced, have something in common so far as vitamin distribution is concerned and appear to constitute a tissue type (135) in the same sense that heart muscle does. Separate papers dealing with the content of individual B vitamins in tumors have been published from the reviewer's and other laboratories (127, 136 to 140).

Relation to psychological functioning.—The idea that each vitamin lack must of necessity cause some specific localized lesion does not have a sound basis, especially in connection with the B vitamins which are necessary constituents of every tissue. Among the effects which may be expected from deficiency of vitamins is a general lack of well-being, which in turn may be reflected in the psychological state of the individual. If methods were developed and applied, it is entirely possible that psychological "lesions" more subtle than the more usual signs of vitamin deficiency could be correlated with improper nutrition, and there can be no doubt regarding the importance of psychological health.

Several studies dealing with B vitamins and on the borderline between biochemistry and psychology have been made in the past year or two. The brain potentials of thiamin-deficient pigeons were found to increase greatly before outward deficiency symptoms appeared (141). Sound-induced convulsive seizures in rats were found to be intimately associated with moderate vitamin B deficiencies. These could be prevented by adequate vitamin supply or by extreme deficiency. Not only thiamin but other B vitamins (also inanition) played a role (142, 143, 144, 145).

In studies of induced human thiamin deficiency at the Mayo Clinic, the importance of mental symptoms has been stressed (146). Gordon mentions "rapid clearing of the mental state" resulting from pantothenic acid administration (73). In other investigations maze test performances by human beings were impaired by deficiency and improved by the administration of thiamin and other B vitamins (147).

Contribution from genetics.—Most biochemists probably have not been expecting important contributions to the study of B vitamins to come via genetics. However, the extremely interesting work of Beadle & Tatum which has resulted in a number of mutant strains of *Neurospora,* each incapable of synthesizing an individual B vitamin (or

other biologically important substance), is a development of the first order of importance (148, 149). Aside from their purely scientific value, these investigations have yielded tools for the assay of known B vitamins and the discovery of entirely new ones.

Species, age, climatic, breed, and individual differences.—That species differences exist with respect to the nutritional requirements for individual B vitamins is well known. The actual content of the tissues is another matter, and so far as present evidence goes, all of the B vitamins are important for all species. There are indications that when the concentration of a vitamin is under physiological control, it is at a higher level than otherwise (11). Relatively little new information about the comparative requirements of different species for the individual B vitamins is available; the findings of individual studies are often given in terms that cannot be directly compared. It would seem that the best basis for comparison of different animals is the amount required for each kilogram of dry food. There is little doubt but that most values expressed on this basis would be of the same order of magnitude, at least for animals of comparable size. A study of the content of milks of different species in eight B vitamins (9) showed that mouse milk is very rich in all B vitamins, and it may safely be concluded that mouse requirements, especially during the suckling stage, are much higher than those of the other animals studied.

The fundamental requirements of mammals for the various B vitamins at different ages are largely unknown, and the problem is complicated by intestinal synthesis which may take place more readily in the adult (81). Studies in progress in the reviewer's laboratory on the vitamin content of tissues at different ages may throw light on this question.

Investigations already referred to (37, 38), as well as others, indicate that requirements for B vitamins may vary with temperature. The loss of substantial amounts of thiamin with perspiration (150) also suggests the possibility of climatic effects. To what extent other B vitamins behave like thiamin in this respect appears to be unknown.

Breed differences in fowls have been found to exist, and the findings not only may be of practical value but are interesting in the light which they may throw on breed (or race) differences among other animals. The requirement of White Leghorn fowls for thiamin has been found distinctly less than that of Rhode Island Red or Plymouth Rock fowls (151). On the other hand, the pantothenic acid require-

ment of Leghorns has been found slightly more than that of Rhode Island Reds (152). F_1-progeny from a cross between Rhode Island Red and White Leghorn showed an intermediate thiamin requirement (151). It is suggested that the existence of different breeds of pigeons may be the potential cause of confusion in thiamin studies, and it would seem far safer in all vitamin studies to specify carefully the breed of animal used. The differences between the content of seven B vitamins in milk from Jersey and Guernsey cows were apparently negligible (9).

The studies on fowls just mentioned suggest that racial differences may exist among human beings and that since individual members of the human family have widely different genetic backgrounds, differences of greater or lesser magnitude should be expected. Even in an inbred colony of experimental animals individual differences in performance occur; on this basis alone the needs of individual human beings for B vitamins might be expected to differ. It is a matter of some practical importance in nutrition and medicine to know how large these differences are. Another matter of some importance in this connection is the question of how long is required to induce deficiencies in human beings. From analogies with animals and consideration of the comparative life spans, one would suppose that it might require several months to produce deficiencies in man. Recently, however, sweeping conclusions with regard to vitamin requirements, involving possible detection of mild deficiencies, have been made on the basis of experiments of six weeks duration (153).

Thiamin

The voluminous accumulation of literature dealing with this vitamin makes impossible an adequate review of the newer advances in a short space.

Functioning in metabolism.—Attempts to determine the definite role or roles played by thiamin in metabolism, aside from its role in decarboxylation of pyruvate, have not met with signal success. In the reviewer's opinion this is due to the fact that cellular metabolism is even more complex than is commonly supposed (all the various B vitamins known, and unknown, and various inorganic ions must be involved), and the picture at best can be only fragmentary at this time. The oxidation of acetate and propionate by propionic acid bacteria which are deficient in thiamin was accelerated by thiamin, especially in the presence of added magnesium and potassium (154). Other substrates behaved differently, and in systems involving living

cells such influences may be very indirect. Studies using deficient rat and chick muscle indicated that increased oxygen consumption induced by thiamin was an indirect effect and that thiamin functions in condensations involving pyruvic acid (155).

Physiological effects.—Subacute thiamin deficiency was found to decrease absorption of glucose, also liver glycogenesis and glycogenolysis (156). It may be that many of the effects of thiamin on animals are indirect in that increased absorption is involved (65). Glycogen in the liver was found to be affected by deficiency and administration of thiamin more than the glycogen in brain, muscle, and heart (157). Interrelations between thiamin and insulin actions have long been suspected but they have not been clarified. Insulin injection like thiamin administration has been found to lower blood pyruvate (158). Restoration of the alkali reserve in diabetic acidosis by thiamin administration has been reported (159). Electrocardiograph changes induced by insulin shock therapy were prevented by thiamin administration (160). An unexpected but well-controlled observation is that polyneuropathy in rats, induced by thiamin deficiency, is markedly delayed by administration of ethyl alcohol (161). A study of lactate-pyruvate relationships in the blood has been proposed as a means of determining the degree of thiamin deficiency (162). Pyruvate oxidation has been found impaired in rats which do not show ordinary symptoms of thiamin deficiency (163).

The thiamin-sparing action of proteins as well as that of fats has been demonstrated and studied (163, 164, 165). Bradycardia was prevented on a high protein diet, but excretion of bisulfite-binding substances was not affected. In this respect the actions of fat and protein differ in that both excretion and bradycardia are affected by fat (164). Fat synthesis was found to be impaired by thiamin, pantothenic acid, or pyridoxin deficiencies, and it was concluded that thiamin does not stand alone as a specific agent concerned with fat synthesis (166).

Thiamin deficiency was found to decrease physical endurance, as measured by tests involving holding out the extended arm and holding the breath, and its administration greatly increased the endurance (167). Fifty mg. of thiamin per day were found to increase the work output induced by glycine and urea administration (168).

The connections between thiamin deficiency and environmental temperature conditions are interesting. The increased thiamin requirement of animals at higher temperatures has already been mentioned

(38). On the other hand, other investigations have shown that neuritis in pigeons is induced and localized as a result of cold conditions and that edema in the feet was caused by a low temperature. Other studies have showed that frost injuries are closely related to thiamin deficiency and that thiamin can act as a preventive for frost bite. Thiamin (also riboflavin) deficiency caused increased susceptibility to pneumococcal infection (169).

The production of anestrus in rats as a result of thiamin or riboflavin deficiency has been referred to earlier (114). An example of another glandular effect has been reported in that the blood calcium was raised by thiamin administration, only, however, when the parathyroids were intact (170).

Massive doses of thiamin (up to 1 gm. per kg. of body weight) have been reported to cause a beriberi-like syndrome in pigeons (171). Pharmacological studies have been made using various substances related to the component parts of the thiamin molecule (172). Acute or mild thiamin deficiency was found to have no pathological effect on the gastric secreting cells in the rat (173). Bradycardia, as observed in thiamin-deficient rats, was concluded to be probably of neurogenic origin (174). An extensive study of the phosphorus combinations in the brains of thiamin-deficient and normal pigeons has been made. The increased inorganic phosphate accompanying deficiency was concluded to be due largely to the formation of hydrolysis products, particularly of adenyl pyrophosphate (175).

Assay methods.—Assaying for thiamin by any but animal feeding experiments involves preparing an extract which will contain quantitatively the vitamin present in the original sample and assaying this extract. While the over-all process, using various methods, has been studied extensively, the quantitative extraction is itself a problem the solution of which cannot be taken for granted.

The thiochrome method has been modified and studied extensively (176, 177, 178, 179) and used extensively in the assay of urine (176, 180, 181), blood (182), vegetable and cereal products (177, 183), and foods (178, 184). Its wide use speaks for its importance, and as long as a suitable extract can be made there is no reason to question its reliability.

The fermentation method has been modified recently by its originators (185) and in two other laboratories (186, 187) and has been used extensively for blood and urine (186) and numerous food materials (185). The method is more difficult and less sure when the

sulfite correction is large. A simplified method has been suggested (188) which, however, is probably of no value except for relatively pure thiamin solutions.

The yeast growth method using a preliminary enzyme digestion for preparing the extract (189) has been applied extensively to the assay of tissues (4, 5, 6, 7), bacteria (8), milks (9), and foods (10). While it has not been compared extensively with other methods, when identical extracts for comparison are used, it appears to be reliable except in the assay of processed foods. In these cases the products of thiamin destruction may function like thiamin and yield erroneous results. Cocarboxylase is not active in the yeast growth method, but thiamin can readily be obtained in a free condition by enzymatic treatment (189). A definite advantage of this method is that it can be applied to very small amounts of tissue containing as little as .0002 μg. of thiamin.

The use of *Phycomyces* as a test organism has its advocates (190, 191), and the method has been used to assay blood (191) with results that are comparable with those obtained by the thiochrome method (182).

A new chemical method (colorimetric) for thiamin, which involves direct treatment of an extract without preliminary adsorption, has been proposed (192). Aside from the fact that it is hardly sensitive enough for some purposes, it appears promising. Like other methods it demands that the vitamin to be assayed be obtained in a soluble form.

Requirements, food sources, etc.—The discovery of Green and co-workers that thiamin deficiency in foxes is induced by feeding raw fish has been confirmed and extended by them (193) and by other investigators (194), but little information is available in addition to that reported in last year's review. Even canned carp can induce thiamin deficiency in trout (195). The latest report is that the agent in raw smelt which causes the destruction of thiamin is not only heat-labile but is destroyed by air drying at room temperature and is extractable by 5 per cent alcohol (196).

Studies of the thiamin content of the average American diet have revealed that before the advent of enriched bread it contained about 0.8 mg. per 2500 kcal. Enrichment of all flour and bread would raise this value to 1.3 mg. Lean pork, bread, and milk are the principal contributors of thiamin to the diet (184). On the basis of extended studies on induced thiamin deficiency in human beings, the minimal requirement is set at 0.55 mg. per 2500 kcal. and for maintenance of

efficiency 1.25 to 1.5 mg. (146). An extensive study has been made of four different methods of determining the nutritional status of an individual with respect to thiamin (197). The four methods showed substantial agreement, and the parenteral administration of 350 μg. thiamin per sq. meter of body area, followed by a determination of the four-hour excretion, was recommended. "Normal" subjects excreted in excess of 50 μg. of total thiamin during the four-hour period after administration. On the basis of balance studies it was concluded that the thiamin requirement of an adult is 0.875 mg. per 2500 kcal. and the recommended intake is 1.25 mg. per 2500 kcal. (198).

Assays of stock animal rations and natural food mixtures suggest that the thiamin content of an ideal diet (suitable for all conditions including pregnancy and lactation) should be over 3 mg. per 2500 kcal. (119). If this is true, widespread mild deficiencies not associated with outward symptoms may exist. Such cannot be detected starting with the basic assumption that everyone is "normal" until proved otherwise.

Riboflavin

Determination.—The two methods of determination which are most favored are the microbiological and the fluorometric. When cereals were treated with taka-diastase, values obtained by the microbiological method were found to agree with those obtained fluorometrically (199). In another laboratory, modifications involving additions to the basal medium made the microbiological method satisfactory (200). Other investigators reported the existence of factors in photolyzed extracts of whole wheat flour and in extracts of rice and wheat bran which stimulate acid production and interfere with the proper evaluation of the riboflavin content of cereal products (201). Both fluorometric and microbiological methods require further study, especially for application to products such as white flour which contain very little riboflavin (202). The fluorometric method has been studied in India both as a method for urine and for tissues (203, 204).

Distribution and requirements.—Fish products—livers, viscera, roe—and fish meals have been found to be good sources of riboflavin (205). Common foods have been assayed (10, 206). A parallel was found between the thiamin and riboflavin contents of cereals and milled products (207). Tumor tissues have been assayed (135, 136). Riboflavin has been found necessary for hatchability of turkey eggs (208), and the requirement for hatchability of Plymouth Rock and Leghorn fowls was found the same (209).

Physiological effects.—The presence of cataract in rats on a deficient diet and its absence when the diets were completely devoid of or were well supplied with riboflavin has been reported (210). Curled toe paralysis in chicks has been found to be due specifically to riboflavin deficiency. Leghorn and cross bred fowls had practically the same requirement (211). Anestrus in rats may be produced or prevented by the withholding or administering of riboflavin (114). Pathological changes due to riboflavin deficiency in mice, including dermatosis and myelin degeneration in the spinal cord, have been studied (212). Pantothenic acid administration has been found to have no effect on the blood or urine riboflavin levels in dogs (213), in contrast to the previous reports with regard to human beings.

One of the most important studies throwing light on the physiological functioning of riboflavin in animals involved the study of the variability of the riboflavin content of the liver of rats as affected by various agents and conditions (64). During digestion and assimilation of food there was found to be a mobilization of riboflavin from other tissues to the liver, even in cases in which the animals had been subjected to prolonged riboflavin deficiency. Thiamin deprivation tended to abolish this mobilization, and pantothenic acid appeared to have some specific function in the process because its administration caused a sharp rise in the liver content.

Miscellaneous investigations.—The decrease in the succinoxidase system in the livers of rats accompanying riboflavin deficiency has been noted (85). A method based upon clearance studies which appears promising for diagnosis of riboflavin deficiency has been proposed (214), and the pharmacology of riboflavin has been studied (215). The lethal dose for rats is high when injected, and oral administration produces no damage.

Light destruction of riboflavin in foods has been found to be rapid and to be influenced by pH and temperature, which are secondary factors (216). Sliced bread may lose riboflavin by being exposed to light under ordinary conditions (207). A water-soluble riboflavin-boron complex suitable for injection has been prepared (217). Riboflavin has been found to be synthesized in the isolated roots of five species of plants (34).

Niacin

Determination. — Colorimetric methods and a microbiological method have been used extensively in studies on niacin. The former

have involved a variety of procedures (218, 219, 220, 221). Extraction procedures have been studied, and it has been found that papain and taka-diastase digestion is effective in most cases. Acid or alkaline digestion converts unknown substances in grains into niacin and thus tends to yield high values (222). The two types of methods give good agreement. Studies have been made of niacin in cereals and cereal products (219, 223, 224, 225), in meats (221), in milks (9, 220), in foods (10, 222, 226), in blood and urine (227), in blood cells and plasma (228), in rat tissues (4), in human tissues (5), in tumor tissues (6, 137), and in bacteria and lower organisms (7, 8).

Niacin deficiency.—Deficiency of niacin in dogs receiving the other known B vitamins has been studied and the daily requirement found to be from 200 to 365 μg. per kg. of body weight. Younger dogs require somewhat more than mature dogs (229). In another study nicotinic acid deficiency in dogs was found not to result in typical black tongue symptoms. Dehydration and electrolyte imbalance, lack of appetite and thirst were outstandingly important. The lives of animals could be prolonged for months by parenteral administration of physiological saline solution (58). Earlier studies (230) had indicated a decrease in the concentration of coenzymes I and II in the tissues of dogs deficient in niacin, but it was doubtful whether this deficiency was the immediate cause of death. In a recent study the niacin content of blood cells and plasma was found unaffected in black tongue and pellagra (228). Another investigation resulted in the finding that in various diseases, including pellagra, the niacin content of the blood does not vary significantly (231). A test for niacin deficiency in man has been proposed on the basis of niacin excretion during twelve to fourteen hours after the administration of 500 mg. Normal individuals were found to excrete more than undernourished or hospital patients (232). A "black tongue" condition in a human patient not thought to be suffering from malnutrition was cured by the administration of niacin (233).

Miscellaneous studies.—Trigonelline and nicotinuric acid have been found to be the principal end products of nicotinic acid metabolism in man and in dogs, and the urinary excretion of these products during deficiency and after dosage with niacin have been studied (234, 235). Further evidence for the synthesis of niacin in the tissues of the rat has been presented and the metabolism of niacin in rats studied. Trigonelline is the chief end product (236). In spite of the reported synthesis of niacin during incubation of hen eggs (89, 90), diets have

been constructed on which chicks respond to niacin (92). Niacin administration was found to cause an increased production of cholesterol in the fatty livers of rats and to diminish the lipotrophic action of choline (43). Administration of niacin to normal individuals is reported to cause a definite but rather slow drop in the blood sugar concentration (237). The bacteriostatic action of various sulfa drugs, in addition to sulfapyridine, was reported to be blocked by nicotinamide and cozymase, and it was concluded that the actions were nonspecific in character (238). The effect of protein on lowering the excretion of niacin has been mentioned (67).

Cozymase and various nicotinamide-containing substances related to it have been found to function as the "V" factor for *Hemophilus parainfluenzae*, but free nicotinamide mixed with other constituents of cozymase is ineffective (239). Nicotinamide was found to be the only factor limiting growth of *Proteus vulgaris* X19 in a synthetic medium. Its functioning in oxidation-reduction was studied (240).

Pantothenic Acid

Specificity.—Supplementing earlier studies, a number of compounds structurally related to pantothenic acid have been prepared and found to be biologically ineffective substitutes, and it was concluded again that pantothenic acid is very specific in its action (241). Ethyl monoacetyl pantothenate and ethyl pantothenate were found, however, to be as effective for rats as an equimolecular amount of calcium pantothenate, and ethyl monoacetyl pantothenate was also tested upon chicks and found to be fully effective (242). These substances are practically without effect on the microorganisms on which they have been tested. These derivatives doubtless yield pantothenic acid in the animal by hydrolysis, and their behavior in this regard is parallel to that of natural forms of bound pantothenic acid which are also hydrolyzed by animals but not in general by microorganisms.

Determination and distribution.—The enzymatic release of the vitamin from meats (243) and other products (189, 244, 245) has been demonstrated and utilized in microbiological assays. Its distribution in a large variety of biological materials has been studied using somewhat modified microbiological procedures (2 to 10, 139, 243, 244, 246). The presence of substances in extracts which interfere with the microbiological tests has been demonstrated and means for

overcoming the difficulties suggested (247, 248). When clarase[1] digestion was used, the values as determined microbiologically approached the higher values obtained by the chick assay method (244). No extensive studies have been made at attempting to correlate the results of the different types of microbiological and biological tests which have been suggested, but it seems probable that a number of methods are capable of yielding useful results.

Fundamental role.—Several attempts have been made to find in what fundamental way pantothenic acid functions in living cells. Its effect on the metabolism of *Proteus morganii* has been studied, and the results suggested that it may have some role in the oxidation of pyruvic acid (248). Pantothenic acid and biotin deficiencies were found to cause a decrease in the capacity of liver tissue to oxidize pyruvate, and it was concluded from the study also that these vitamins are probably components of enzyme systems which are active in connection with pyruvate metabolism (86). A study of the effect of pantothenic acid on yeast maceration juice preparations led to the conclusion that it probably is not a dissociable coenzyme involved in any of the recognized steps in carbohydrate metabolism (249). Administration of glucose to rabbits was found to cause a lowering of the pantothenic acid content of the blood (250). This suggests a connection between the vitamin and carbohydrate metabolism.

Deficiencies, requirements and physiological effects.—Considerable information is already available regarding the requirements of fowls and the effects of deficiencies. A recent study indicates that the pantothenic acid requirement of Leghorn chicks is less than half what had been previously found by Jukes, but this contradictory finding cannot be accepted without further verification (153). Evidence has been presented which demonstrates rather convincingly that the "vitamin B_3" which entered into the vitamin studies prominently a few years ago was principally pantothenic acid (117).

Further extensive study has been made of the pathological effects of pantothenic acid deficiency in rats (55). Prominent results were kidney, adrenal, and cardiac damage, and dehydration of the tissues. The dehydration and the observed increased appetite for salt were probably associated with the impairment of the adrenals. In another investigation it was found that the production of "blood-caked" whisk-

[1] This is the designation under which the Takamine Laboratories sell what was formerly called taka-diastase.

ers in rats—a condition previously observed to result from pantothenic acid deficiency—could be induced by water deprivation, in which case pantothenic acid was ineffective as a cure so long as water was withheld (57). The interrelations between pantothenic acid, pyridoxin, and unsaturated fat acids in rat nutrition have been mentioned (61, 62, 63).

The requirement of rats for pantothenic acid has been found to decrease with age (251). This, however, may be due largely to difference in intestinal synthesis (81). An increased requirement during thyroid feeding has been observed (115). Pantothenic acid appears to have a specific function in rats in connection with the mobilization of riboflavin to the liver (64). In line with previous observations on increased hatchability of hen eggs resulting from pantothenic acid feeding, litter size in rats and mice has been found to increase as a result of an increased supply of this vitamin (252).

Pantothenic acid deficiency in dogs has been carefully studied and described (253). Dogs may eat normally up until a day or two of their death. The organs most affected in the case of dogs are not the same as in the rat. The adrenals were relatively little affected, but the kidneys were, and severe lesions were found in the stomach and intestines. The livers were mottled and had a high fat content. The requirement of young puppies was estimated at 100 μg. of calcium pantothenate per kg. of body weight per day. Adult dogs needed less.

Administration of either pantothenic acid or riboflavin to dogs was found not to affect the excretion of the other vitamin (213). Pantothenic acid deficiency was found to cause a lowering of the blood lipides in dogs and to induce the production of fatty livers (254).

Several studies have been described during 1942 dealing with pantothenic acid deficiency in hogs (255, 256, 257). This leads to slow growth, rough coat, loss of hair, emaciation, and ulcers in the intestinal tract, and a "goose stepping" gait (associated with nerve degeneration). As with the dog, gastrointestinal symptoms were prominent.

The importance of pantothenic acid as an anti-gray hair factor has been emphasized by several recent studies. Feather depigmentation in black fowls has been observed as a result of pantothenic acid deficiency (258). Pantothenic acid has been found to prevent graying in rats induced by a heated diet, and *p*-aminobenzoic acid was found ineffective (259). In another study pantothenic acid cured pattern grayness (260).

Two new values for the average daily human excretion of pantothenic acid have been recorded which agree substantially with previous results (147, 261) and indicate that the daily excretion by "normal" individuals is about 3.5 mg. Two independent estimates of the pantothenic acid requirement of man agreed at about 10 mg. per day (119, 147). Both values were based upon indirect evidence. Pantothenic acid appeared to cause a prompt response in five cases of peripheral neuritis, in one case of Korsakoff's syndrome in a male alcoholic, and in two cases of delirium tremens, all of which had failed to respond to other B vitamins (147, 262).

Miscellaneous investigations.—Increased intestinal digestion and absorption in dogs have been observed to result from pantothenic acid administration, even when the animals had been receiving an adequate diet (66). Intestinal production of pantothenic acid in rats has been studied (80), and indications were found that it is decreased in cecectomized rats (81). Pantothenic acid administration has been reported to promote intestinal synthesis of inositol in mice (76).

The inhibiting effects of pantoyltaurine and related pantothenic acid analogues on bacteria have been determined. Only pantoyltaurine, pantoyltauramide, and homopantoyltaurine are strongly inhibitory, and these are effective primarily on organisms which require pantothenic acid in the culture medium (95, 98, 99, 107). Other analogues showed some inhibitory action on organisms not requiring pantothenic acid in the culture medium, presumably by interfering with biological synthesis. The biological synthesis of pantothenic acid by yeast has been studied (263). Its requirement by nonsporulating anerobes of intestinal origin has also been investigated (18). A mold mutant incapable of synthesizing pantothenic acid had been produced (264).

Pyridoxin—Vitamin B_6

Contrary to the common impression, the chemistry of vitamin B_6 is in a highly unsatisfactory state. There is no question, of course, regarding the fundamental chemistry of pyridoxin, that pyridoxin occurs naturally, or that pyridoxin has vitamin properties. The serious question is whether the vitamin B_6 activity of tissues is due solely to pyridoxin or whether there are other substances (probably closely related) which serve equally well or better. There is far more uncertainty on this point with respect to pyridoxin than there is in the case of any of the four B vitamins already considered.

Crystalline pyridoxin hydrochloride (m.p. 205°) was isolated from B_6 concentrates in several laboratories almost simultaneously. Short notes were published and details were lacking. No data were given to indicate that anything more than a fraction of the B_6 activity of an extract was obtainable in the form of crystalline pyridoxin. György's report in the form of a communication was most complete (264a). After the first few steps of concentration had been carried out, the material was 5 to 25 per cent pure, and the yield of the activity was 10 to 30 per cent of the original. Further losses during purification were not recorded. Assay methods were not conducive to accuracy since they were dependent upon cure of acrodynia. Pyridoxin is evidently relatively easy to isolate; it was obtained as a by-product by Japanese workers in 1931, at which time it was not recognized as a vitamin (264b).

The only reasonable interpretation of recent studies (mostly microbiological in nature) is that there is in tissue extracts at least one other substance, temporarily called pseudopyridoxin which has vitamin B_6 properties and which may account for a substantial amount of vitamin B_6 activity (265). For yeast, pseudopyridoxin apparently has the same physiological activity as pyridoxin, but for certain lactic acid organisms, pseudopyridoxin is a thousand times more active. Its activity for animals is unknown, but it may well be between the two extremes. There appears to be no previous evidence contrary to this interpretation, and the complications which exist make it impossible either to know precisely what natural vitamin B_6 is or to obtain satisfactory evidence regarding the distribution of the active agents. These facts have a profound effect on almost all research dealing with vitamin B_6. The fact that some animals, at least, have a capacity for transforming pyridoxin into pseudopyridoxin simplifies the picture in one way and complicates it in another, unless this capacity can be assumed to be unlimited.

Assay methods—distribution.—An adaptation has been made of the Scudi colorimetric method for pyridoxin (266), but in view of the remarks above, its specificity may be questioned. This is also true of the Swaminathan colorimetric method (267). The rat growth method as applied to meat and meat products (268) constitutes, in the reviewer's opinion, the best method for determining vitamin B_6 although unknown vitamins cannot be ruled out completely. All of the above methods give much higher values than the yeast growth method for pyridoxin (269).

The use of *Lb. casei* in the assay for pyridoxin (36) cannot be justified in light of what is now known regarding pseudopyridoxin and its high potency for *Lb. casei*. The presence of pyridoxin in conjugated form in rice bran concentrates has been reported (270), and the existence of a conjugate not available to yeast may explain in part the low assay values obtained using yeast. Even if this is so, this does not, however, invalidate the evidence regarding pseudopyridoxin.

Animal experiments.—Unlike rats, mice are reported to give no growth response to pyridoxin (271). Pyridoxin deficiency is reported to occur without dermatitis (272). The interrelations between pantothenic acid, pyridoxin, and fat acids have already been mentioned (61, 62, 63). A convulsive syndrome in rats due to pyridoxin deficiency has been reported from two laboratories (273, 274). Pyridoxin-deficient dogs and rats have been found to excrete in the urine a substance capable of yielding a green pigment (275, 276). Storage of pyridoxin in rats has been investigated (277), and its metabolism in rats and man has been followed (278).

In an earlier German report pyridoxin was shown to be much more effective than other B vitamins tested in the alleviation in rabbits of anemia due to bleeding (279). The anemia in dogs resulting from pyridoxin deficiency has recently been studied, and a lag in the results of pyridoxin therapy can be overcome by administering liver extract (280). The pyridoxin requirement of chicks has been found to be about 300 μg. per 100 gm. of feed (281), and its necessity for maintenance of the nervous tissue and prevention of anemia in hogs has been reported (257).

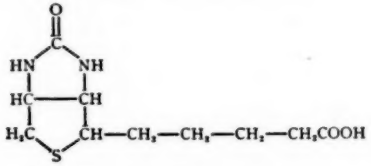
Pyridoxin administration has been reported to have a beneficial effect and to cause reduction in the oiliness of the skin in certain cases of acne (282).

Experiments with plants.—The growth of excised tomato roots has been found to be stimulated by the addition of pyridoxin (and thiamin) to a basal mineral sugar solution (283). The fungus *Graphium ulmi* was found to require pyridoxin as a nutrilite before noticeable growth took place (284); other known vitamins had little effect.

Biotin

Structure.—The announcement of the structure of biotin as determined by du Vigneaud and his collaborators (285) was certainly a leading event in the vitamin field in 1942, not because of the satisfaction we may derive from writing the formula for one more vitamin,

but because biotin synthesis is now possible, and what has probably been the most costly chemical ever placed on the market will now become vastly cheaper. We cannot take space here for discussion of the whole biotin problem or even the immediate steps leading to the announcement of the structure indicated below:



I. Biotin (free acid)

Availability of this vitamin in adequate amounts will now make possible definite solution to a good many hitherto difficult problems.

Bound forms—assays.—The problem of the bound forms of biotin which occur in tissues has been the subject of several studies (286, 287, 288). No single procedure will with certainty release biotin from all its natural combinations without destruction, but strong acid hydrolysis is usually effective. Avidin, the protein constituent of egg white which combines with biotin, has been obtained in crystalline form (289). Its stability has also been studied (290). Microbiological assays for biotin, involving *Lb. casei* as the test organism, have been described (36, 291) and its distribution studied (2 to 10) using the yeast method.

Physiological effects.—Several investigations have been reported dealing with biotin deficiencies and requirements in fowls (292, 293, 294, 295). There is substantial agreement that less than 1 μg. per day per chick is required to prevent deficiency. Dermatitis, poor feathering, slow growth, and perosis are symptoms of deficiency which are overcome by biotin administration.

Biotin deficiency symptoms in rats have been further studied (296, 297) and a typical paralytic symptom described. The leg muscle creatine in paralytic rats was high and was reduced by biotin administration.

Production of human biotin deficiency by feeding raw egg white and its cure with biotin concentrates have been reported (298).

Pharmacologically biotin appeared in several tests to be inactive (299). Its possible relationship to pyruvate metabolism has been men-

tioned (25) and its relationship to cancer discussed (6, 125, 126, 127). The production of biotin deficiency by administration of bacteriostatic drugs has been mentioned (78, 79). Biotin production by bacteria has been studied quantitatively (8, 74).

Lower organisms.—Evidence has been presented that biotin is a nutrilite for *Diplodia macrospora* (300), various *Rhizobia* (301), for the fungus *Marasmius androsaceus* (20), and for insects (26). For fungi and bacteria it may be a growth requirement or it may be only stimulative.

Inositol

Distribution.—The status of inositol as a member of the "B family" of vitamins is strengthened by studies of its distribution, which indicate that like other members of the family it is universally present in living matter (2 to 10). It is more abundant in tissues than other members of the family, and its distribution appears to be somewhat erratic.

Animal experiments.—The finding that pantothenic acid induces the intestinal synthesis of inositol in mice helps considerably to clear up previous, contradictory reports (76). It would appear that pantothenic acid may be necessary for the absorption of inositol since it is reported that inositol deficiency may result even when inositol is in the diet, if pantothenic acid is absent.

A convincing and clarifying study has been made in which it was found that Rockland strain black rats perform just as satisfactorily on a diet containing six addenda—thiamin, riboflavin, niacin, pantothenic acid, pyridoxin, and choline — as when both inositol and *p*-aminobenzoic acid were added also (77). When either one of the latter two was added alone, the rats suffered and a syndrome was precipitated which could be cured only by the addition of the other. The requirement for *p*-aminobenzoic acid, for example, could be demonstrated regularly only when inositol was in the diet. An interpretation of these findings based upon differences in intestinal synthesis was not as convincing as the experimental findings themselves. More recently new evidence has been presented to show that both inositol and *p*-aminobenzoic acid, especially the former, are important for lactation in rats (302).

Four types of combination in which inositol occurs naturally have been pointed out, including a new brain phosphatide (303, 304). During incubation of hen eggs it was found that inositol synthesis does

not take place as was previously thought, but instead inositol is freed from combinations which are not available to the test organism (yeast) (91). Experiments to determine whether inositol is required by growing chicks will be awaited with interest.

p-Aminobenzoic Acid

Antagonism to sulfa drugs.—The antagonism of *p*-aminobenzoic acid to the bacteriostatic effects of sulfanilamide and related drugs, which served to point to the existence of *p*-aminobenzoic acid as a vitamin, continues to be studied (100 to 109). Substances other than *p*-aminobenzoic acid can act antagonistically to the sulfa drugs (108), 109). Sulfanilamide can itself act as a growth substance for bacteria (110) or for animals (111). The specificity of *p*-aminobenzoic acid in antagonizing sulfanilamide is manifestly limited. One investigator has claimed that *p*-aminobenzoic acid acts as a narcotic similar to urethane (305), but evidence has been presented to show that this interpretation is not justified (107).

Animal and human experiments.—*p*-Aminobenzoic acid has been found to be ineffective in the prevention of graying of rats on synthetic and heated diets (259). The presence of inositol in a synthetic diet was found to be necessary in order to demonstrate the effectiveness of *p*-aminobenzoic acid as an anti-gray hair factor for rats. The presence of *p*-aminobenzoic acid, on the other hand, was necessary to demonstrate the anti-alopecia effect of inositol (77).

Evidence has been presented to indicate that *p*-aminobenzoic acid is important, in addition to inositol and the other known B vitamins, in lactation and rearing of young. *p*-Aminobenzoic acid without inositol delayed the secretion of milk at first, and it is possible that this may have been due in part to the heavy doses used, 15 mg. per day. Sulfanilamide serves as a partial substitute for *p*-aminobenzoic acid in the diet of rats (111).

Additional clinical reports of the benefits of *p*-aminobenzoic acid administration have been published. Fertility in women and pigmentation have been reported improved (306).

Miscellaneous investigations—assay methods. — *p*-Aminobenzoic acid serves with biotin as one of two growth factors required by *Clostridium acetobutylicium* (307). It has also been found to be a nutrilite for *Acetobacter suboxidans* (308). A microbiological test for *p*-aminobenzoic acid has been devised using the latter observation as a basis (309). The discovery of a mold mutant which is unable to synthesize

p-aminobenzoic acid and which requires it for growth is the basis for another quantitative test (149). The development and use of assay methods which have hitherto been lacking should yield interesting fundamental information regarding the quantitative distribution of this vitamin. A hint of its possible functions results from the observation that it affects tyrosinase activity (310).

Unidentified Vitamins Associated with the "B Family"

Another member of the "B family" of vitamins might be added to the recognized list, namely, folic acid, were it not for the fact that its isolation in pure form has not been reported. It has been obtained in sufficiently concentrated form so that it is physiologically active in concentrations comparable with that of any vitamin except biotin. It occurs in all tissues and single-celled organisms tested (2 to 10); is freed from them by autolysis or enzyme action (189); is an effective nutrilite for a number of yeasts and lactic acid bacteria and for tetanus organisms (17, 311); and has been shown to have vitamin properties when fed to rats which have impaired intestinal synthesis due to feeding succinyl sulfathiazole (79).

There are many investigations during 1942 and in previous years which point to the existence of B vitamins in addition to those known at the time the investigations were planned. There are practically no reported animal experiments, however, indicating definitely the existence of B vitamins in addition to the nine already discussed, namely, thiamin, riboflavin, niacin, pantothenic acid, vitamin B_6 (pyridoxin), biotin, inositol, *p*-aminobenzoic acid, and folic acid. Whenever a new vitamin is discovered and its status established, all work involving possible new factors must be repeated anew, eliminating the new member as a possibility. There are a number of factors for which clear-cut evidence of existence has been presented, which, however, can conceivably be made up of biotin, inositol, *p*-aminobenzoic acid, folic acid, or some combination of the members of this group.

Among the "unknowns" which have been studied during 1942 or late 1941 are chick factors (312 to 317); the grass juice factor (318); lactation factors L_1 and L_2, Bx (319, 320) (inositol and *p*-aminobenzoic acid are doubtless involved); new factors required by mice (12, 321); unidentified factors required by dogs (322); new factors required by guinea pigs (13); a trout anemia factor (32); and an anticancer principle (129).

Among the known compounds for which vitamin or vitamin-like properties have been claimed are muscle or yeast adenylic acid, reported effective for human patients (323), anthranilic acid (324, 325) which is reported to stimulate the growth of certain yeasts and to be an anti-anemia agent, and cozymase and related substances which are nutrilites for *H. parainfluenzae* (239).

Unrecognized substances affecting the growth of bacteria or fungi include pseudopyridoxin (265), a growth factor for hemolytic streptococci (326), factor Z_1 (327), a new heat-labile *Lb. casei* factor (247), a plasmoptyzate growth factor for *Colpoda* (23), factors required by *Ceratosmella* (19) and by *Erysipelothrix* and *Listerella* (328).

Ascorbic Acid

It is somewhat discouraging that the functions of a vitamin which has been known as long as ascorbic acid should remain enshrouded in pretty much the same baffling uncertainty as was true four years ago (329). Very little that is fundamentally new regarding it has been uncovered during 1942.

Determination—distribution.—Numerous papers dealing with ascorbic acid determination have been published (330 to 341). One is an ultramicromethod (330); another uses resting bacteria to convert dehydroascorbic acid to the reduced form (334); others involve determination in the presence of sulfur dioxide (333, 342). Among the newer rich sources of vitamin C are conifer needles (a potential winter source) (342). An important series of Russian studies on the effects of processing foods on their ascorbic acid content has been published (343 to 347). The importance of the potato as a source of ascorbic acid has been stressed again (348). It is unfortunate that some writers on the subject make the tacit assumption that the ascorbic acid content of the diet is measured in terms of the amount of citrus fruit juice used. For lower income people the potato is of highest importance in this connection.

Miscellaneous investigations.—The effects of ascorbic acid supply on guinea pigs have been studied, particularly with respect to the adrenals (349, 350, 351), to tubercular infections (352, 353), to the distribution of phosphatases (354, 355) and other enzymes (356), and to tissue respiration (357). Relationships between hormones and ascorbic acid have been studied (358 to 362) and ascorbic acid synthesis by tissues *in vitro* has been investigated (363), as has biosyn-

thesis in plants and animals (364). New investigations on the oxidation of ascorbic acid have also been made (365, 366).

The clinical reports dealing with the use of ascorbic acid are entirely too numerous to mention in a short review.

The saturation method for determining an individual's status with respect to ascorbic acid has been found satisfactory (367), and the requirements of young children (368) and adults (369) have been studied using this method. A simple method for the same purpose, which has found continued use, involves the intradermal injection of 2,6-dichlorophenolindophenol (Rotter) and observance of the decolorization time (370).

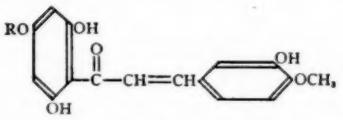
One of the more interesting observations, because of its fundamental implications, is that in some cases of C avitaminosis a test dose may be excreted which more or less completely indicates an inability to utilize the vitamin (371). A careful study of ascorbic acid tolerance indicated that there may be wide variation of deficiencies and that mild deficiencies which are far removed from clinical scurvy can be determined and studied (372). Other reports emphasize the importance of C deficiencies which are not accompanied by clinical symptoms (373, 374). The association of gastric lesions with low ascorbic acid reserves has been reported (375). This might mean a specific direct connection between the pathological state and the ascorbic acid supply but it might only indicate on the part of the individuals a general neglect of modern nutritional principles. Irresponsive cases of pernicious anemia have been reported to be greatly benefited by ascorbic acid administration (376, 377). Hay fever has also been reported to respond to ascorbic acid but no control group was used nor were objective criteria established (378).

Vitamin P

The evidence for the existence of vitamin P has grown materially stronger since last year's review was prepared. A Russian study involved a comparison between the effects of ascorbic acid (plus citric acid) and a comparable amount of lemon juice when fed to guinea pigs for three months. Extravasation of blood, changes of the vasoconstriction behavior, and several other differences appeared in the animals obtaining pure ascorbic acid (379). Groups of women on different levels of vitamin C intake were studied, and their capillary fragility was not altered by vitamin C administration. It was

supposed that vitamin P was lacking in the vitamin C-free diet used (280).

A somewhat abbreviated but important paper was published reporting the isolation of a new oxidation-reduction enzyme containing vitamin P as the prosthetic group (381). The active principle of vitamin P is thought to be hesperidin chalcone, represented below, in which R is a sugar group.



II. Hesperidin chalcone

This is capable of being transformed (reversibly) by reduction and ring closure to the flavanone glucoside which contains a benzopyrone nucleus, and it is supposed that the protein isolated in impure form is an enzyme which is effective in oxidation-reduction reactions. The chalcone was found in preliminary experiments to decrease capillary fragility and prevent hemorrhages. A review concerning vitamin P has been published (382).

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FAT-SOLUBLE VITAMINS[1]

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Vitamin A

Baxter & Robeson have continued (1) with the preparation and study of crystalline vitamin A and its crystalline esters (2), and the compounds have been assayed[y2] biologically against U.S.P. Reference Oil II, the assays being on a provisional basis for reasons explained in the next section. Baxter has followed the work started by Holmes & Corbet (3) who produced vitamin A containing methanol of crystallization; and of Hamano (4) who prepared pure vitamin A-β-naphthoate. The method of making the solvent-free crystals of free vitamin A was as follows: A rich fish liver oil containing more than 100,000 units of vitamin A per gm. was concentrated by molecular distillation giving a fraction having an absorption coefficient of $E^{1\%}_{1\,cm.}$ (328 mμ) = 500. This was saponified in an atmosphere of nitrogen and again distilled. This treatment yielded a red viscous oil—$E^{1\%}_{1\,cm.}$ (328 mμ) = 1450—which could be crystallized from ethyl formate at −35°. Repeated crystallizations gave vitamin A—$E^{1\%}_{1\,cm.}$ (328 mμ) = 1780—which could not be further purified by chromatographic adsorption. The crystalline vitamin was esterified with acetyl chloride and other acid chlorides in the presence of pyridine. A sample of Mead's (5) β-naphthoate was found to be identical with that prepared by Baxter. This suggests that both were pure. The properties of the compounds are summarized in Table I. The melting points and purity require checking against similar preparations from other laboratories before they can be accepted as absolute values. The table affords the following provisional deductions: (*a*) The extinction coefficient of free vitamin A is slightly higher than that of the vitamin moiety of the aliphatic esters, acetate and palmitate. (*b*) For a corresponding vitamin A content the biological potency of the aliphatic esters is the same as that of the alcohol. (*c*) The "conversion factor," Po-

[1] Communication No. 46 from the Laboratories of Distillation Products, Inc.

[2] Superscript *y* indicates material available to the author which has not yet been published.

TABLE I

PROPERTIES OF CRYSTALLINE VITAMIN A COMPOUNDS
[Baxter, Robeson, Harris (Mead)]

Vitamin A Compound	Crystal Habit	M.P.	Elimination Maximum from Glyceride CYO†	$E^{1\%}_{1cm.}$ (328 mμ)	Equivalent $E^{1\%}_{1cm.}$ (328 mμ) Calculated for Vitamin A Content	With $SbCl_3$ $E^{1\%}_{1cm.}$ (622 mμ)	Bio-Potency U.S.P. Units*		Conversion Factor: C.F. = Potency/ $E^{1\%}_{1cm.}$ (328 mμ)
							Actual	Potency of A-Content	
A-OH	Long yellow prisms	64°	....	1,750	1,750	4,800	4,300,000	4,300,000	2450 ± 227
A-acetate	Compact primrose yellow prisms	57–58°	....	1,510	1,730	4,580	3,520,000	4,040,000	2350 ± 133
A-palmitate	Irregular pale yellow plates	27–28°	213°	940	1,720	2,535	2,310,000	4,230,000	2520 ± 183
di-A-succinate	Pale yellow branching prisms	76–77°	<260°	1,240	1,420	4,450	3,140,000	3,590,000	2630
A-β-naphthoate	Pale primrose plates and clumps	74–75°	....	1,090	1,640	2,940	3,440,000	5,290,000 ((2,225,000)*)	3160 ± 145

* Assay by Underhill and Coward.

† CYO means constant yield oil.

tency / $E^{1\%}_{1\,cm.}$ (328 mμ), of the crystalline alcohol is the same as the vitamin A in its aliphatic esters. (*d*) This conversion factor is significantly higher than that adopted commercially in the United States (2000) and is higher than that of the vitamin A in most fish liver oils. (*e*) The spectral absorptions of the vitamin A moieties of the β-naphthoate and succinate esters (esters which do not occur in nature) are out of line with the others; the biological potencies are abnormal; consequently the conversion factors are divergent in what seems to be a more or less accidental manner.

Bioassay and conversion factor of vitamin A.—Among the many papers on this general theme during the past eighteen years, three or four stand out because they set forth important, yet seemingly irreconcilable points of view. There is the reasonable belief of the spectroscopist (6) that a unit of absorption at the peak of a characteristic band should indicate a fixed number of vitamin molecules which, in turn, should have a fixed curative value. This assumption does not accord with facts (7). Vitamin extracts show factors ranging from 1000 to 2000, vitamin oils, from 1200 to 2400. In a classic paper which refutes a uniform factor, Morgan and associates (8) listed cod liver oils having absorption / potency relationships varying over a range of 1.0 to 2.8. Wilkinson (9) has recently shown that variation in constituents of the depletion diet will alter the response from a given oil by two to one. These workers know that in extreme cases where two equal "spectral doses" are assayed simultaneously—the same oil on different diets or different oils on the same diet—one will fail to support life, the other furnish a flourishing group of rats.

An equally classic publication, the 1935 Report on "The Standardization and Estimation of Vitamin A" (10), was inspired by the opposite point of view: that the discrepancies were due to uncertainty of assay and that, given a large enough cooperative assay among a sufficient number of laboratories, the discrepancies could be averaged away. The anomalies left over were to be rationalized by the statistical mathematicians. This begged the question of what the vendor or user was to do with a consignment of vitamin concentrate that consistently refused to show the bioassay predicted by the spectrograph. The minority opinion of the Morgan-Wilkinson school remained unchanged but the majority continued using multiple assay (11). Faith in averages was exemplified in the blending for an International Standard of "Halibut Liver Oil from Parke, Davis with a sample from Aberdeen" (12). No less than 1665 rats in fourteen lab-

oratories were used recently (13) to compare U.S.P. Reference Oil II with β-carotene, and apparent variations in potency of from 1000 to 2500 units per gm. were encountered though the concurrent limits of vitamin D assay were within 100 to 130 units per gm.

The early multiple assays were justified because they offered the only hope of rationalizing the unexplainable. Indeed, these great experiments afford some of the best evidence today that there are real differences in oils between the potency predicted from spectral absorption values and that found by biological assay. In recent years it has become clear that the discrepancies must be understood and compensated rather than rationalized.

The first ray of light, still unperceived by many workers with vitamin A, especially amongst physicians, was the observation by Moore (14) and Davies (15) that ingestion of extra vitamin E increases the storage of vitamin A in the liver of the rat, increases the time taken to deplete a rat of vitamin A, and delays the onset of dental symptoms attributable to vitamin A deficiency. Davies and Moore foresaw the implication of their discovery:

> The tocopherols are higher substituted members of the class of hydroxy aromatic substances of which hydroquinone is a lower and less hydroxylated member . . . . often used for stabilizing solutions of vitamin A in fats. The tocopherols may possibly have a similar action under physiological conditions. . . . . Any factor such as vitamin E deficiency which causes secondary vitamin A deficiency in experimental animals must be worth consideration in the investigation of human diseases . . . .,

and we might add, of vitamin A assays.

This important work at first received less attention than it deserved, perhaps because other investigators (16) could confirm the phenomenon only at high levels of vitamin E. This was evidently due to the vitamin being fed as the acetate of the synthetic *d,l*-form which, though active as a preservative in the liver and tissues after hydrolysis and absorption, is relatively inactive in the intestines. Only the minor effect after absorption was thus observable. When approximately 30,000 units of vitamin A were fed over sixty days with 0.9 mg. *d,l*-α-tocopherol acetate given daily to one half of the isogenic group of rats, the liver stores on autopsy were found to be: with vitamin E, 3365 I.U. of vitamin A; without vitamin E, 2570 I.U. of vitamin A.

In Rochester, Moore's observations at once struck a sympathetic note because vitamin E was involved in the manufacture of distilled

concentrates (17) and was eventually identified as a natural constituent of fish liver oils (18). To the group in Rochester it seemed important to extend the work and demonstrate, if possible, not only increased storage of vitamin A in the liver following ingestion of vitamin E, but enhanced growth and vitality on a suboptimal intake of vitamin A (19). A fuller discussion of the work is given under the heading, "Vitamin E," but we will relate here those parts necessary for the present argument.

The usual bioassay technique for vitamin A was made the basis for the test, but the diet was altered so as to deplete the animals of both vitamins A and E. Most of the vitamin E in the standard U.S.P. XI depletion diet is derived from the specified 5 per cent of vegetable fat which by common consent is generally purified cottonseed oil. Substitution of olive oil produced a diet which was satisfactorily low in vitamin E without introducing the complications (20) incurred by treatment with ferric chloride (21). From experiments completed previously with the unmodified A-depletion diet containing Wesson Oil, quantities of vitamin A, A-acetate, and β-carotene were found which generally gave weight gains of from 10 to 20 gm. in twenty-eight days. The rats were then divided into three large groups, one for each kind of vitamin A. The main groups were broken up into subgroups, successive members of which received the previously chosen quantity of vitamin A but increasing quantities of vitamin E. In this way the effect of tocopherols on the utilization of vitamin A was tested over a wide logarithmic range covering all practicable dosages. Some typical data are shown in Table II. From these and other results it has been

TABLE II

EFFECT OF DIFFERENT QUANTITIES OF VITAMIN E ON THE GAIN IN WEIGHT ACCRUING FROM FIXED QUANTITIES OF VITAMIN A

	Amounts of Tocopherol Supplement Fed						
	0 mg.	.025 mg.	.05 mg.	.15 mg	.5 mg.	1.5 mg.	5.0 mg.
	Gain in Weight of Male Rats in 28 Days						
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Vitamin A, .46 μg.....	+ 8.6	....	20.7	28.8	27.6	31.7	....
Vitamin A-acetate, .5 μg.	− 6.3	+ 4.0	....	13.0	....	25.0	....
β-carotene, .79 μg.....	−22.0	−23.0	−21.0	+10.3	29.3	22.7	12.3

deduced: (*a*) Vitamin E exerts a sparing action on two forms of vitamin A and to a greater extent on β-carotene. (*b*) Arbitrary choice if a single level of vitamin E might have failed to show an A-E synergy or might have suggested an antagonism. (*c*) Male rats show a greater variation than female. (*d*) β-carotene at optimum E-intake becomes an efficient source of vitamin A for the rat, being more than half as active as the free vitamin. This suggests, though it does not yet prove, that the conversion of β-carotene *in vivo* is by fission (22) rather than by an oxidation (23) destructive to one end of the molecule.

There is thus ample evidence that vitamin E assists vitamin A in a large but variable manner. What about substances antagonistic to vitamin A? Do they exist in practical assay and nutrition? The answer is definitely in the affirmative though less systematic investigation has been done in this field. Robinson (24) has shown that oxidized vitamin has less activity than its spectral absorption would indicate. Sherman (25) has found that linolenates and linolates were antagonistic to moderate amounts of carotene. Palm oil and decolorized butter fat (26) depress carotene activity to a third. Rancid fats (27) or stale diet (28) are especially destructive to the A vitamins. Unsaturated glycerides contained in linseed and fish oils occupy a special position in the synergy-antagonism picture because they are essential to growth (29, 30) and can increase the weight gain (25) otherwise attributable to vitamin A, yet they readily form peroxides destructive to the vitamin in the gastro-intestinal tract. The muscular dystrophy induced in herbivora by these fats (31) may be due in part to the destruction of vitamin A in the muscle tissue.

The above evidence—and work cited later—suggests that the major seat of the syn-anti-effects is in the stomach and intestine. Quackenbush and associates (32) have examined the action of soybean distillates and other substances, including hydroquinone, on carotene in the diet and have come independently to the same conclusion:

> The evidence indicates that in promoting a biological response to carotene the tocopherol functions as an antioxidant in the gastrointestinal tract rather than as a vitamin regulating some phase of metabolism in the tissues.

Their experiments are doubly significant because they illustrate the activity of very small quantities of vitamin E.

We may now assemble the difficulties in the way of establishing a universal conversion factor and procedure for bioassay:

(*a*) Animal assay measures the molecular content of vitamin A

in food, multiplied by a utility coefficient, generally less than unity, which we will call ψ. The factor ψ is dependent on the chemical form of the vitamin A, the presence of protective or destructive agents and substances such as phospholipids which aid intestinal absorption, and, of course, on the kind of diet[3] and the proportion of edible fat.

(*b*) Biossay measures, in addition to vitamin A, the active carotenoids and all substances curative for vitamin A deficiency.

(*c*) Spectral absorption rarely measures the true quantity of vitamin A. The extinction coefficient, $E^{1\%}_{1\,cm.}$ (328 mμ), like the animal response, varies with the nature (n)[4] of the vitamin, i.e., -alcohol, -palmitate, or -β-naphthoate; and the spectral purity (p) is a measure of whether other substances, such as anhydro vitamin A and subvitamin A of little or no activity, are absorbing in the same region. Now the experimentally found conversion factor (c.f. = potency/extinction coefficient) can be written: biological units = $E^{1\%}_{1\,cm.}$ (328 mμ) × c.f. To obtain the true conversion factor, C.F., we must evidently expand to:

$$\text{Bio-units} \times \psi = E^{1\%}_{1\,cm.}\ (328\ m\mu) \times \text{C.F.}\ (n \times p)$$

$$\text{or C.F.} = \frac{\text{potency}}{\text{extinction}} \cdot \frac{\psi}{n \times p}$$

It is this group of subsidiary factors, $\psi/n \times p$, that causes trouble. The spectral contaminants (p) are not serious in high potency oils though in halibut liver, salmon, or Mexican shark oils of high acid value, vitamin A_2, anhydro vitamin A, and opaque fatty acids may cause serious errors.

Much remains to be learned concerning the dependence of ψ on the state of chemical combination (n) of the vitamin A. It is probable that the wide range of conversion factors encountered by Morgan (8) in different cod liver oils arose from different distribution of the fatty acids. Again, it is possible that there are isomers of vitamin A which have potencies different from the usual variety and that these isomers are concentrated in the "soluble vitamin A" fraction (34) of Baxter and associates. It should be noted that while crystal-

[3] The controversy between Ramasarma (33) and Wilkinson (7), while unsettled, does not favor the former since he used such large supplements of carotene, twenty times the minimum, that the utilization of this provitamin could scarcely have been the factor limiting the growth of the rats.

[4] Symbols used provisionally for illustrative purposes; an exact mathematical equivalence is not expressed or intended.

line free vitamin A is more potent, molecule for molecule, than the vitamin A in the parent fish liver oil, commercial saponified concentrates are generally less potent (35), having conversion factors ranging from 50 to 85 per cent of the factor for the unsaponified oil (36, 37).

β-Carotene and a possible new international standard.—By far the largest known disturbing factor, and one modifying ψ, is tocopherol. Refined cottonseed oil contributes on an average 0.2 mg. of mixed α- and γ-tocopherols to the U.S.P. A-depletion diet daily and thus provides a supplement in the most sensitive region of the E-carotene synergy. A change in concentration of ±100 per cent, which is an everyday occurrence between purchased samples of oil, gives an expected variation in growth response per 28 days, as shown in Table III.

TABLE III

VARIATION OF GROWTH WITH VARIATION OF TOCOPHEROL IN COTTONSEED OIL*

Tocopherol mg.	Vitamin A		Carotene	
	Low Dose	High Dose	Low Dose	High Dose
0.00	12	25	−10 to −20	20
0.1	25	45	10	34
0.2	28	45	18	37
0.4	29	45	25	44
0.8–1.0	30	44	27	44

* Change in body weight expressed in grams.

Occasionally a commercial sample of cottonseed oil is found to contain no vitamin E. If this has been lost by rancidification, the destruction of carotene will be doubly aggravated. The only safe way to retain carotene as a standard would be to add 0.6 mg. of tocopherols to the diet daily or bring the content of the vegetable oil up to 0.06 per cent ± .01 per cent, which is on the plateau of the A-E synergy curve. But assays done with this adjustment would require one and one-half to two times as much vitamin A to equal the growth then induced by the carotene. Applied to the restandardization of U.S.P. Reference Oil II, for instance, the U.S.P. Unit would become nearly double in size, the numerical value of all pharmaceutical and scientific dosages would require halving, and commerce in the A vitamins would be disorganized pending revaluation of inventory and

contracts. Carotene is evidently out of the question as a continued standard.

At first sight, the remedy would seem to be to designate as the new standard a quantity of crystalline vitamin A, or its acetate, having approximately the same curative values as the U.S.P. Unit, or the International Unit, or the average. A provisional recommendation could be, for instance, U.S.P. standard vitamin A-depletion diet with 5 per cent of peroxide-free cottonseed oil adjusted to contain 0.08 to 0.1 per cent tocopherols (unesterified). A revised International Unit would consist of: 0.23 μg. crystalline vitamin A, or 0.27 mμ vitamin A-acetate. But complications arise. Most of the vitamin A in America, perhaps 100 trillion units a year, is drawn from shark, halibut, and dogfish liver oils. These oils and their distilled concentrates represent the great bulk of the vitamin A of commerce and they are all satisfactorily represented by a factor of 2000. The crystalline materials have factors around 2400, that is, 1.2 times larger in simultaneous assays, and are thus not strictly comparable.

Even when the standard is chosen, the question arises of using the E-fortified test diet for the commercial test sample. Two rival samples, one containing vitamin E and one contaminated with rancid fat, might both assay favorably, whereas the first would give the consumer full value in vitamin A activity, the second would not. The standard and its surrounding problems must be settled by further research and considerable discussion by the proper committees. The matter has been ventilated here in the hope of discouraging a plethora of giant cooperative assays before all the variables have been named.

Substances related to vitamin A.—The study (38) of a compound similar to or identical with vitamin A_2 is being continued with attempts to isolate the pure substance from pike liver oil.

Le Rosen (39), Zechmeister & Schroeder (40) describe interesting classes of compounds which are isomeric with certain known carotenoids. A C_{40}-hydrocarbon, separated from the tangerine-tomato, has an absorption 35 mμ lower than lycopene and is designated prolycopene. The chromophore contains from five to seven *cis*-double bonds in contrast with the usual *trans*-configuration. Another compound provisionally named pro-γ-carotene, extracted from the palm fruit *Butia capitata,* is a stereoisomer of γ-carotene and contains from four to five of the eleven double bonds in *cis*-configuration. The material reverts to an equilibrium mixture of itself with γ-carotene on treatment with iodine.

Kitol.—A remarkable compound, provisionally named kitol (from ketos, a whale), has been extracted from whale liver oil (41). This oil, which has been used in large quantities by those countries with whaling fleets, has been considered a poor source of pure vitamin A because of its complicated absorption spectrum and low conversion factor, 1000 to 1200. One of the contaminating substances has an absorption band at 290 mμ and has been given some study by Edisbury (42) and Willstaedt (43) and their respective co-workers. Believing that the chromogen at 290 mμ might be subvitamin A (44) or a new as yet uncharacterized compound, Embree & Shantz (41) subjected the oil to intensive study. The desterolated nonsaponifiable fraction was treated in the cyclic molecular still to remove free vitamin A, anhydro A, and other contaminants of low molecular weight. The residue containing the substance kitol, which absorbs at 290 mμ, was again subjected to saponification and the kitol again appeared in the extract. It was further concentrated by adsorption from petroleum ether on alumina and elution with ether-alcohol. The final product, apparently pure, had the following characteristics:

Appearance—Viscous pale yellow oil above room temperature, a solid glass at 25° C.

Probable Formula—$C_{40}H_{55}(OH)_2$ Calc. Carbon 83.9 per cent, Hydrogen 10.48 per cent. Found Carbon 83.4 per cent, Hydrogen 10.11 per cent

Molecular Weight—Theory 572, found 575

Emmerie-Engel Value—$E^{1}\%_{1\,cm.}$ (290 mμ) = 580

$SbCl_3$ Product—$E^{1}\%_{1\,cm.}$ (428, 505, and 580 mμ) = 228, 162, and 104

Vitamin Potency—Zero (much less than 140,000 units per gm.)

Elimination Maximum—225° C.

Occurrence—As fatty acid esters in whale and other liver oils

Optical Rotation—$\alpha^{25°}_{546.1\,m\mu} = -1.35°$

Attempts to distill kitol unchanged failed, but the loss of yield was compensated by the appearance in the distillate of vitamin A which had not been in the original kitol concentrate. Thus, kitol, which was biologically inactive, acquired vitamin A activity and an absorption at 328 mμ on heating. Kitol is, therefore, a provitamin A of a new kind and the first provitamin A of any kind to be changed into the vitamin by laboratory means.

Kitol appears to be a divitamin A containing two hydroxyl groups though only one molecule of vitamin A can be recovered on pyrolysis. The elucidation of its exact structure must await further research.

When whaling is again resumed, the subsequent conversion of kitol will add a significant fraction to the world supply of vitamin A. The discovery is of even more significance to the chemistry of the A vitamins and to their biochemistry. That kitol occurs most plentifully in the whale is suggestive. Fishes, as a class, require or tolerate more vitamin A than mammals. A mammal, such as the whale, confined to a marine diet, may well be embarrassed with too much vitamin A which it might detoxify by converting two molecules to one of inactive kitol. However, kitol has been found in the livers of oxen and sheep which are not overburdened with supplies of vitamin. Etiologically it would seem that kitol is a post- rather than a provitamin. This designation is strengthened by the occurrence of kitol$_2$, corresponding exactly with vitamin A$_2$, whereas no unique provitamin A$_2$ is known.

Anhydro or "cyclized" vitamin A.—A substance which occurs in traces in all vitamin A-bearing oils and is formed whenever the vitamin is mistreated has long been referred to as "cyclized" vitamin A, following Heilbron's (45) analysis of the products formed from the selenium dehydrogenation of the vitamin. Further research (46) has shown that the reaction is one of simple dehydration, probably without cyclization. The substance, which is generally prepared by treating a dilute alcoholic solution of the vitamin with anhydrous hydrochloric acid, can also be formed by the refluxing of the natural esters with alcohol [Cawley (47)]. Microhydrogenation measurements together with the ultraviolet absorption spectrum suggest the presence of six conjugated double bonds or five conjugated double and one allene bond. Robeson has recently prepared crystals of the material (well-formed bright yellow rhombs), the composition of which is given in Table IV [cf. (46)].

TABLE IV

ANALYSIS OF ANHYDRO VITAMIN A

	Carbon	Hydrogen
Experimental	88.57	10.43
Anhydro Vitamin A, $C_{20}H_{28}$, requires	89.55	10.45
"Cyclized" Vitamin A, $C_{20}H_{30}O$, requires	83.92	10.49

The absorption peak of the antimony trichloride reaction product of anhydro vitamin A is not, as generally believed, identical with that of vitamin A but is displaced from 2 to 3 mμ towards the red. The value $E^{1\%}_{1\,cm.}$ (620 mμ) is 5500, in contrast with 4800 found for vitamin A (1).

Isoanhydro vitamin A and subvitamin A.—Anhydro vitamin A is formed in a few minutes from the vitamin in alcoholic hydrochloric acid. If the reaction mixture is allowed to stand for from twelve to sixteen hours, the three characteristic absorption bands of anhydro vitamin A disappear and give place to new bands at 330, 350, and 370 mμ; these belong to a substance which is isomeric (48) with anhydro vitamin A.

Subvitamin A (44) occurs to a small extent in most liver oils and is responsible, with kitol, for the distortion of the ultraviolet absorption curve on the short wave length side of the maximum at 328 mμ. It is not readily detected or separated as such but it yields an anhydro product which has markedly different solubility and adsorption properties from anhydro vitamins A_1 or A_2, and can, therefore, be determined chromatographically. Neither subvitamin A nor anhydro subvitamin A has vitamin A activity.

The compounds just described occur in small quantities and play no prominent part in the current vitamin A picture; their significance lies in emphasizing the complexity of vitamin A chemistry and the pitfalls in the way of placing too great reliance on the ultraviolet or antimony trichloride absorption spectra of products containing vitamin A.

Estimation of vitamin A.—Ultraviolet absorption measurements continue to be employed for the commercial evaluation of fish liver oils and concentrates for vitamin A, using the quartz spectrograph or one of the many new photoelectric comparators (49). These have also been adopted for the Carr-Price reaction for measurement of vitamin A extracted from foods and pharmaceuticals. The Corning Glass Company has joined the Kimbal Glass Company in the manufacture of colored (50) laboratory glassware needed for handling dilute solutions of the vitamin.

The Tswett adsorption column continues to be the most important tool in preparation of carotenoid and vitamin A mixtures for analysis. A notable advance in technique (51) has been made using an adsorbent wetted with a liquid which is insoluble or partly soluble in the solution treated. In one case, silica gel wetted with water was used with chromatograph solutions in chloroform; degrees of separation corresponding to 20 to 30 unit treatments have been reported. Dicalcium phosphate (52), activated by treatment with alkali and subsequent drying, has been found to be an excellent adsorptive agent for separating chlorophyll and xanthophyll from β-carotene. A mixture of

magnesium oxide and silica has been used to purify solutions of tomato extract in benzene for β-carotene analysis by Mackinney (53) and others; they have also given comprehensive directions for analysis of various fruits and vegetables for lycopene, cryptoxanthin, and carotenes. The absorption spectra of zeaxanthol, luteol, and neozeaxanthol have been determined after extraction from yellow corn (54). The pigments have been separated by the use of immiscible solvents and the degree of separation checked by chromatography. The ratio of the pigments is found to be relatively unaffected by the number of genes for yellow color (55) in the original corn. The technique of extraction of β-carotene from mixed feeds is described by Seshan & Sen (56) who employ alkaline extraction in aldehyde-free alcohol, followed by solvent partitioning and estimation in the Lovibond instrument against a dichromate standard. Neo-β-carotene has been separated from β-carotene by chromatography and the two compounds found to be reversibly (57, 58) interconvertible.

The intensity of optical absorption of vitamin A and the position of the maximum in the ultraviolet spectrum have long been known (59, 60) to be affected slightly by the nature of the solvent. Morgareidge (61) has shown that when the usual polar solvent (ethanol) is replaced by a less polar liquid such as cyclohexane, the absorption maximum of most preparations examined shifted near to the accepted wave length of 328 mμ. U.S.P. Reference Oil II was an exception and is thus a poor spectrographic standard. Careful absorption studies of pure crystalline vitamin A and its palmitate (62), which show the fine structure of the peak of the curves under different conditions of aging, have been made in various solvents, including ethanol and cyclohexane. The value $E^{1\%}_{1\,cm.} = 1721$ at 328 mμ for the uncombined vitamin in ethanol is a trifle lower than reported by Baxter & Robeson (see Table I), but the values at 326 mμ are in near agreement.

Estimations of β-carotene excreted in human and animal stools have been continued. Wald *et al.* (63) recommend grinding the fresh feces with anhydrous sodium sulfate prior to extraction with *n*-heptane, saponification, and partition between petroleum ether and methanol. The carotenoids remain in the petroleum while free vitamin A passes to the methanol. Eriksen & Höygaard (64) extract the wet feces with methanol and benzene; Harris[9] freezes the feces in dry ice, reduces the frozen material to a powder, extracts an aliquot with benzene, and collects the water in a graduate from which the fecal dry weight can be calculated (64a). The final extracts must invariably be

chromatographed or otherwise purified (65, 66) before colorimetric determination. The quantity of vitamin and provitamin A recovered, subtracted from the quantity ingested, has hitherto been assumed to represent the quantity absorbed. Since tocopherol increases the quantity excreted as well as the biological potency of that retained, it is evident that a third figure, the quantity destroyed in the intestine, must be added to balance the equation. Until further work has evaluated this factor, figures for the intestinal absorption of vitamin A and carotenoids will be untrustworthy. Survival in the feces ranges from 2 to 4 per cent of vitamin A intake to 40 to 99 per cent of carotene intake.

The formation of β-carotene by intestinal bacteria (67) has been observed and members of the vitamin B complex have also been found in the gastrointestinal tract (68). The possibility of intestinal biogenesis further complicates the intake-discard-utilization balance of all the vitamins.

The absorption and retention of vitamin A and provitamin A by the domestic hen have been studied by Russell (69) and others. On a low-fat ration the quantity of yellow pigment excreted was more than the carotenoids eaten. The pigment was not β-carotene though normal diet permitted excretion of pigments rich in carotene. The apparent carotene excreted in the latter case varied from 15 to 88 per cent of that ingested. Between 32 and 40 per cent of carotene given intravenously to birds on a normal diet appeared later in the feces, whereas none appeared on a low-fat intake. The fecal excretion of free vitamin A was not appreciably influenced by the fat content of the diet though liver storage was severely impaired on a low-fat intake. The critical part that tocopherol must have played and the possible role of fat as a source of tocopherol or vehicle for absorption were not taken into consideration. The figures for retention of vitamin evidently require correction by subtraction of intestinal losses, as yet unevaluated. The paper contains a wealth of information that can be reinterpreted later.

The conversion of carotene and the utilization of vitamin A supplements by the cow, as evidenced by increased vitamin output in milk, have attracted several investigators. Cows fed from ¾ million to 2 million units of vitamin A esters daily in the form of shark liver oil yielded milk with a vitamin A content of 1570 to 2035 I.U. per quart (70). The optimum vitamin A intake was 900,000 units daily. In other experiments on Guernsey cows (71), where 700,000 units were fed daily as shark liver oil, the total recovery in the milk was only

3 per cent. From experiments on the rat (72) it would seem that carotene is utilized less efficiently from alfalfa meal (21 per cent) than when dissolved in cottonseed oil (59 per cent), doubtless through greater intestinal destruction in the former case. Dried alfalfa is seldom a sufficient source of carotene for the cow (73) as the quantity of provitamin in the steam-dried meal is found to vary between 5 and 225 p.p.m., the higher figure being one third of the optimum required. Factors contributing to low vitamin content (28) of dried alfalfa are conditions of dehydration and storage. The state of aggregation of the alfalfa, whether loose or in compressed pellets, is unimportant compared with low temperature and exclusion of air during storage. Only 10 per cent loss was encountered in six months at —23° in air. The carotene content of common feedstuffs is dependent on the mode of culture (74) and correct reaping. Destruction after harvesting could be eliminated almost completely by dehydration in the absence of air and light, the latter alone accounting for from 50 to 85 per cent of the loss (75, 76).

Studies of carotene and vitamin A balance (77) in cattle have shown that the vitamin content of blood plasma is the most sensitive criterion. More than 10 μg. of carotene per 100 ml. of blood plasma should be present in the growing calf; Holstein yearlings should show from 70 to 150 μg., and Guernseys, from 110 to 114 μg. The minimum vitamin A requirement of the young fox is said to be from 15 to 25 I.U. per day, but liver storage does not occur until four times as much is fed (78).

Physiological response.—The essential physiological function of vitamin A is still obscure but much light has been thrown on the conditions modifying the function. How the vitamin is absorbed, where it is stored, and how transported and eliminated or destroyed are fairly well understood. Three general methods of investigation are in favor. Direct analysis of the organs and blood plasma by the antimony trichloride reaction have yielded a mass of related information in the hands of Clausen (79) and McCoord (80); inspection of tissue through fluorescence microscopy by Popper (81) and others (82) has shown in what part of the organs the vitamin resides. Visual threshold photometry by Hecht (83), Wald (84), and others (85) has traced the correlation between vitamin A and vision (86). Only incidental reference will be made to visual studies since authoritative reviews have appeared during the year (87, 88, 89). Attempts to relate practical vitamin therapy with the supposed visual function of vitamin A

have proved unreliable, and little improvement of normal night vision has been reported from high vitamin A therapy. In what follows we shall quote largely from Clausen's address (90) to the Gibson Island Vitamin Conference in 1942 and from his notes gathered for the 1943 Harvey Lecture.

Vitamin A and carotene are absorbed through the intestinal wall and scarcely at all through the stomach. Absorption is preceded by fission or hydrolysis in the small intestine, and it is the free vitamin that is transferred across the gut wall (91). Esterification is immediately re-established (92) with the original acids in the case of abnormally high dosage of vitamin A ester, or with acids representative of body fat under normal circumstances (93). The passage across the gut is facilitated by bile (94) and safeguarded by antioxidants (32), while transfer to final destination is by both lymphatics and blood stream since ligation (90) of the thoracic duct does not impose much hindrance. The greatest single reservoir is the liver where the vitamin exists as a relatively high concentration of esters, eventually mobilized via the blood stream as free unesterified vitamin. Under high vitamin dosage or after taking much alcohol (95, 96) the blood content of vitamin A is enriched from the intestine or by leaching of the liver and tissues, but the extra vitamin is merely in passage as the ester and is believed to exert no immediate biological function. In fact, the content of the active, free vitamin A is depressed somewhat by excessive liberation of esters.

If a vitamin A is a metabolic intermediate or catalyst, it does not appear to be used up in the process of metabolism; the weight of evidence favors accidental destruction. The vitamin is not eliminated in the urine in normal health (97) and only to a minor degree in fecal fat (63, 98). The rate of disappearance is not accelerated by thyroid extract (99) or healthy increase of metabolism, but it is destroyed rapidly in high fever. This destruction is sometimes dramatically retarded or repaired by vitamin E, which suggests that both normal and abnormal wastage are due to thermally promoted oxidation[v]. The destruction of whatever vitamin A is available is accelerated by conditions of vitamin A deficiency, which throws some light on the rapid change of response in the usual rat assay to decreasing increments of the vitamin.

The intestinal absorption of vitamin A and carotene depend on bodily health and the condition of the food. In cases of failure to supply pancreatic enzymes to the duodenal contents, the vitamin esters

are not hydrolyzed and absorption suffers. Absorption suffers also in celiac disease or wherever absorption of dietary fat is impaired. Storage is lessened in all liver derangements and excretion occurs in kidney disease. The absorption of carotene (100) is dependent on the thoroughness of digestion of vegetable tissue. Raw carrot (64) yielded 1 per cent of its carotene as body vitamin A while cooked carrot gave 35 per cent; the figures for spinach were 45 per cent and 50 per cent, respectively. Many observers believe that while carotene is convertible into vitamin A, it is a separate vitamin in its own right (101). Attempts to deplete certain individuals of vitamin A and carotene and the lack of dependence of visual threshold on vitamin A ingested are believed to be due to intestinal flora which manufacture carotene (67, 102).

The fluorescence technique demands special mention though an excellent survey has recently appeared (103). The whole organ, or a slice of tissue fixed in formaldehyde (82), is illuminated by a source of ultraviolet light from which all visible rays have been screened. If vitamin A is present, the specimen glows for a few seconds with green fluorescence which, through the microscope, is seen to proceed from oil droplets. Tissues containing vitamin A_2 show droplets with a reddish glow. In the hands of Popper this technique has revealed much of the metabolic path of vitamin A in health and disease. A particularly striking photograph (104) shows the diffusion of vitamin A through the intestinal mucosa of the rat. Under normal circumstances the vitamin is stored in oil droplets in the normal liver cells, but under high intake the vitamin invades the Kupfer (105) cells which become brilliantly fluorescent. It appears to be these cells which can dispose in a month or so of a quantity of vitamin A, predicated by Moore (14) to be sufficient to last the lifetime of the rat. The fluorescence technique, applied to human skin (106), reveals no vitamin A in the epidermis even after high vitamin therapy, which accounts perhaps for the long interval between therapy and healing in epidermal vitamin A deficiency. Popper has found that diets deficient in choline (107), which induce fatty livers, rob the liver of vitamin A even though large quantities of the vitamin are fed. Some of the vitamin is excreted in the urine but much is stored in the kidney. Vitamin storage is reduced and urinary excretion initiated in many kinds of disease, notably in tuberculosis (108) and liver derangement. Comparative figures for vitamin A content of normal blood and blood from patients suffering with Laennec's (109) cirrhosis are given as 198 and 65 I.U.

per 100 ml., respectively. In liver injury of the rat (110) caused by dibenzanthracene (111), liver storage of vitamin A is depressed while the kidney acquires abnormally large quantities.

A more positive type of experiment in vitamin A therapy has been instituted by Straumfjord (112) who claims that relatively enormous doses of vitamin A confer clinical benefits not associated with normal vitamin A therapy. The benefits have been experienced by persons with persistent follicular hyperketosis of the arms, persistent and abnormal corns, warts, and callouses, all of which have disappeared after a few weeks or months on a daily intake of 100,000 units of vitamin A in the form of a rich fish liver oil. In a recent paper (113), it is shown that 30 mg. of mixed tocopherols taken daily with the fish liver oil increase the vitamin A content of human blood plasma four times more than achieved by the fish liver oil alone. Much clinical data is being assembled concerning the output of factory workers fed these large vitamin A supplements, but it is too early for the benefits expected by the author to be analyzed, confirmed, and accepted. The benefits claimed include unusual freedom from colds, a conclusion which is at variance with the findings (114) of many years concerning moderate vitamin A and D supplements. The belief that high vitamin A therapy (115, 116) may provide a cure for renal hypertension (117) may be abandoned in favor of other as yet unidentified substances contained in marine oils (117a).

Vitamin D

During a search for 22,23-dihydrobrassicasterol in rapeseed oil, Fernholz & MacPhillamy (118) found an isomeric 28-carbon sterol melting at 157–158° which they named "campesterol." This has been converted by Ruigh (119), following an earlier lead (120), into a new provitamin D (7 = dehydrocampesterol) which when activated by the quartz mercury vapor lamp has a potency of about 4 million units per gm. towards the rat—with potency to poultry unreported. The conversion of the sterol to the 7-dehydro compound did not follow Windaus' (121) method of removing one molecule of benzoic acid from a sterol dibenzoate (122) but required instead the removal of one molecule from the monobenzoate of 7-hydroxycampesterol. Other means (123) of affecting the conversion of sterols have been described.

A preparation containing dihydrotachysterol$_2$, available under the commercial name (124) of A.T.10, has been studied for some years as an antitetanus remedy owing to its power of raising serum calcium

in hypoparathyroid and postoperative conditions (125). The material is prepared by reduction of the 3,5-dinitro-4-methyl-benzoic acid ester of tachysterol (a partially irradiated ergosterol). There have been conflicting reports (126) as to the antirachitic potency in clinical tests (127) and towards rats, figures from 0 to 100,000 I.U. per gm. being mentioned (128). A recheck by Correll & Wise (129) shows that the rat potency is about 30,000 units per gm., but the relative potency towards chicks is higher than ever reported in the chemistry of rickets. If quantities, different according to absolute potency, of calciferol (D_2), 7-dehydrocholesterol (D_3), cod liver oil, and A.T.10 be chosen which have equal healing for the rat, then the relative multiples of these quantities needed to heal the chick are approximately: D_2, 50; D_3, 1; cod liver oil, 1; and A.T.10, ¼.

Chemical analysis of the D vitamins has not progressed but there have been modifications of bioassay. The toes (130) of chicks, in place of the tibia, have been suggested for ash determination on grounds of cleanliness and possibility of retaining the live animal. Removal of epiphyseal cartilage (131) from the tibia also contributes to cleaner specimens. The shortening (132) of the twenty-one day period of the A.O.A.C. assay results in decreased sensitivity. A new assay has been proposed using radiostrontium (133) in lieu of tagged calcium. The sensitivity is low but the response varies linearly over a wide logarithmic range of dosage, which makes the method of promise in exploratory work. The strontium is fed to the rats maintained in cages especially constructed for the study of metabolism and the activity of the urine and feces measured before and after giving a single vitamin D sample. Strontium excretion is decreased drastically within two days, thus providing a quick estimation.

Destruction of vitamin D in feeds (134), fortified milk powders, and other vehicles has been reported (135). Of milk samples collected by one group (136) 17 per cent were below labeled potency. Little difference has been noted between the ability of various kinds (137) of vitamin D to withstand oxidation and dead storage. Sugar and kaolin were especially harmful while cereal (138) and ground seed products were protective during storage.

Soon after the discovery (139) that equal rat units of calciferol and cod liver oil were unequally curative for chick rickets, it was accepted by default that members of the growing list of D vitamins were interchangeable in mammalian rickets, while specific in avian rickets. Only recently have qualitative differences been sought in clinical prac-

tice. Important studies have been made on the incidence of dental caries in children. During an eight-month period, McBeath & Verlin (140) found that school children receiving no extra vitamin D developed an average of 4.5 carious surfaces; those receiving 800 units daily of cod liver oil vitamin D, 1.65 surfaces; those taking 800 units of viosterol, 2.48; and those receiving 3200 units of viosterol daily, 2.77 surfaces. A smaller experiment conducted by Livermore (141) on Ojibway Indians showed reduction of caries to 32 per cent when the children were transferred from their usual to a carefully balanced diet; to 16 per cent when this diet was accompanied by the vitamin-mineral supplement "visyneral"; and to 27 per cent when the uncontrolled diet was augmented by "visyneral." It has been found elsewhere in earlier experiments that massive doses of calciferol (142) can practically eliminate dental caries in children from two to sixteen years of age.

These clinical experiments are heralded as a forward step in prophylactic dentistry, but a grave warning is issued (143) against too hasty an adoption of their findings. The superiority of vitamin D_3 was demonstrated in only one of the three surveys and is at variance with the general experience of pediatricians that D_2 and D_3 are interchangeable. The dental benefits accruing from fantastic doses of D_2 may be offset by long-time metabolic disturbances and do not yet warrant revision of the 500 to 1000 units a day recommended for children.

Vitamin E

Many details have been added to the chemistry of the tocopherols, chromans, and coumarins during 1942, particularly by Smith and collaborators. A new synthesis has been devised (144) which provides apparently unequivocal proof that α-tocopherol has a chroman rather than a coumarin structure. Three new synthetic dimethyl-ethyl-tocols have been described (145) with antisterility activities comparable with β- and γ-tocopherols. An amino derivative (146) and the calcium half succinate (147) of α-tocopherol each retains the full activity of the parent vitamin and each should prove to be a valuable tool for research. Papers with Kolthoff and others have described the electrode potential (148), the redox characteristics (149), and analysis by the dropping mercury cathode (150) of substances related to vitamin E (151). These studies will form a mine of information when the details of the true physiological function of vitamin E come to be elucidated. Baxter

& Robeson (152) have prepared a number of pure crystalline tocopherol derivatives which have been tested by Joffe & Harris (153) using the Mason (154) and Bacharach (155) modification of the Evans-Burr assay technique (156). The physicochemical properties of the tocopherols and derivatives are listed in Table V. As pointed out by Harris, it will be seen that the natural β- and γ-tocopherols are of lower relative potency than hitherto claimed (153, 157, 158). All three tocopherols exhibit (159) nearly equal vitamin A and carotene synergy (19); the average mixture, as distilled from vegetable oils, shows a slightly increased potency (see page 382).

Bioassay.—It follows from the last statement that the three tocopherols exhibit different potencies according to the biological effect measured. Equally active as intestinal sparing agents and having unknown relative curative powers for the muscular dystrophies, they respond unequally to the rat antisterility test by which they are usually assayed.

The modifications of the assay technique (154, 155) now in general use can be operated with a degree of precision which compares more than favorably with current determinations of vitamins A and D. Some laboratories administer the test substance in ten equal doses beginning the first day of pregnancy; others use three doses given on the fourth, fifth, and sixth days; and still others give a single dose on the first day. Autopsy is on the sixteenth day, or the animals may be allowed to bear their young normally. Variations of ±20 per cent in dose will drastically alter (154) the ratio of viable young to dead and resorbed. However, while the comparative mean fertility dose (m.f.d.) can be determined to within ±10 per cent, the dosages observed in different laboratories may vary over a 3:1 range (160, 161, 162) showing that some factor, such as the efficiency of absorption of the vitamins from the gastrointestinal tract or utilization afterwards, must differ greatly. Using the terminology which we developed for vitamin A, the E vitamins would have a very variable ψ-factor. If Q denotes the real quantity of α-tocopherol, then $Q_\alpha \cdot \psi_\alpha = \text{m.f.d.} = 0.55$ to 1.71 mg. in various laboratories (163). $Q_\alpha \cdot \psi_\alpha = 0.8$; $Q_\beta \cdot \psi_\beta = 2.0$; and $Q_\gamma \cdot \psi_\gamma = 9.0$ in our laboratory. The question arises as to whether ψ is similar for all three compounds with Q equal to 0.8, 2.0, and 9.0, or whether Q is nearly constant with ψ varying; or whether both vary. The practical outcome of the interlaboratory variation is the need for a comparison standard which, since the introduction of synthetic *d,l*-α-tocopherol (164), has generally been the commercial

TABLE V

PROPERTIES OF TOCOPHEROLS

Tocopherol	Habit	M.P.	Absorption Maximum Wave Lengths in mμ	Absorption Coefficients $E^{1\%}_{1cm.}$	Persistence	Relative m.f.d. (Tocopherol)†
Synthetic α-(Merck)	Viscous oil	.....	292	74.7	6.8	1.0
Synthetic β-(Merck)	Viscous oil	.....	297	85.1	7.3	5.0
Synthetic γ-(Merck)	Viscous oil	.....	300	89.2	9.2	>20.0
Synthetic α-acetate (Hoffmann-LaRoche)	Viscous yellow oil	.....	286	41	3.8	1.0*
Synthetic α-palmitate (Merck, Baxter)	White granules	36–38°	286	30.9	7.8	...
Natural α-(Baxter, *et al.*)	Viscous oil	.....	292	73.7	17.5	1.0
Natural β-(Baxter, *et al.*)	Viscous oil	.....	297	87.6	20.6	2.5
Natural γ-(Baxter, *et al.*)	Viscous oil	.....	298	92.8	18.0	12.0
Natural α-acetate (Robeson)	Needles	26.5–27.5°	286	41.2	8.2	1.0
Natural α-acid succinate	Needles	76–77°	286	38.5	5.7	1.0*
Natural α-palmitate	Glossy laths	42–43°	286	26.8	5.7	12.0*
Natural β-azobenzene carboxylate	Orange rosettes	69.5–70.5°	328	414.0	3.6	8.0*
Natural γ-palmitate	White glossy laths	44–45°	286	39.0	4.4	12.0*
Natural α-allophanate	White granules	157–158°	286	33.6	5.6	...
Natural β-allophanate	Colorless laths	138–139°	286	44.5	5.0	...
Natural γ-allophanate	White granules	136–138°	286	46.0	5.9	...
Synthetic α-calcium succinate	White solid	210–212°	...	....	...	1.0*
Synthetic α-phosphate (sodium salt)	White powder	.....	...	....	...	1.0*
Synthetic α-tocopherylamine (hydrochloride)	White solid	156–158°	...	....	...	1.0*

* On basis of tocopherol content.

† m.f.d. = mean fertility dose

acetate (165). Recently this compound has been adopted as an official standard (163) and subjected to cooperative assay with the result that 1 mg. has been designated as the "international" standard unit (166). The physical constants corresponding with the segregated supply,[5] including extinction coefficient, Emmerie-Engel and Furter-Meyer values (on the saponified material), have not yet been published. In view of slight variations between samples of liquid *d,l*-α-tocopherol acetate, it would seem better to redesignate as standard the crystalline natural or *d,l*-acetate or succinate, the latter being recommended for its high activity, stability, ease of purification, and hence, duplication. In endorsing a pure vitamin E compound as an official standard, the Committee (163) renders a service, for the existence of a yardstick allows the antisterility activity of unknown mixtures of tocopherols to be given numerical rating. Unfortunately, the rating is for an artificially induced syndrome in the rat and is not applicable to the more general covitamin E role played by the three tocopherols in general nutrition. The designation of α-tocopherol acetate as a standard is welcome as long as it does not prevent the functions of the other tocopherols in free or esterified condition from receiving their proper appreciation and, thus, degree of investigation.

Physicochemical assay.—The chemical determination of vitamin E is in an unsatisfactory state of development though there have been recent improvements in methods. The Furter-Meyer test registers all substances which give yellow-red colors with nitric acid. The silver nitrate (167) modification may give only a partial discrimination between α-, β-, and γ-tocopherols. The Emmerie-Engel method with dipyridyl registers all bodies which reduce ferric chloride. Spectrographic assay is hampered by general absorption at 293 mμ and by derivatives of vitamin A.

An analysis (168) first devised for vitamin K (169) measures vitamin E in fresh and oxidized condition with some promise of specificity. To determine the tocoquinones, the extract is reduced by hydrogen in the presence of a potential indicator, mixed with 2,6-dichloroindophenol, and the diminution in blue color measured. Determination of total tocopherol is preceded by an oxidation with gold salts. The method has revealed the facts, important if substantiated, that

[5] Obtainable from the Department of Biological Standards, National Institute of Medical Research, Hampstead, London, N.W. 3, England; or from Professor J. Fullerton Cook, Pharmacopoeia Revision Committee, 43d Street and Woodland Avenue, Philadelphia, Pennsylvania.

† m.f.d. = mean fertility dose

• On basis of tocopherol content.

from 4 to 16 per cent of the tocopherol in vegetable oils is in the quinone form while blood plasma of man and dog contains a half-and-half mixture.

All the analytical methods in trained hands can be used successfully for chemical investigation and manufacturing control, but they fail when applied directly to most foods and animal tissues. Intermediate purification must be resorted to. Devlin & Mattill (170) in a comprehensive review condemn saponification and recommend chromatography; Andrews (171) requires both processes. Cobler & Viergiver[9] find saponification followed by mild hydrogenation causes little destruction to vitamin E while abolishing the carotenoids completely (172). It is well recognized that serious losses may occur during extraction and saponification, particularly if an inert atmosphere is not provided. The operator thus has the choice of accepting a too-high value on unpurified material, a true tocopherol reading on purified material which has suffered unknown losses, and a biological determination which reveals the tocopherol content as modified by the absorption factor ψ, performed on an aliquot of the complete sample or its extract. Hence, knowledge concerning the true vitamin E content of human and animal diets is meager and a column for vitamin E values is missing from most recent tables of food values. In an effort to learn the probable limits of content of tocopherols[9] (159) in a one-day allowance of a relatively rich human diet, including eggs, meat, vegetables, etc., an analysis has been made by the various methods listed earlier on the bulked foods, taken directly from the dining table and dehydrated under high vacuum before extraction with solvent. The Furter-Meyer test showed a red color equivalent to 167 apparent mg. of α-tocopherol; the Emmerie-Engel test showed 12.7 mg.; spectral assay showed general absorption interpreted as 6 mg.; while bioassay showed 4 mg. of true α-tocopherol. The content of mixed tocopherols of the day's food was thus 4 to 8 mg. of mixed tocopherols. Much more data are required to establish the average vitamin E content of diets of various income brackets.

Vitamin E deficiency in animals.—The histopathological changes associated with lack of vitamin E, especially on synthetic diets purified with chemicals (21) which may produce concurrent deficiencies (157), have been explored exhaustively during the past fifteen years. Autolysis of the developing embryo in the female rat (173) and degeneration of germinal epithelium of the testes of males were among the first conditions recognized. In the past year Mason (157) has described a

new criterion of vitamin E deficiency in the developing (16-day) embryo, namely, the appearance of large areas grossly infiltrated by red blood and thus made visible. The lesions are characterized by vasodilation, congestion, and vascular stasis of superficial channels, particularly in the head, shoulders, and trunk. Dam and others have continued observations (174, 175) on alimentary exudative diathesis in chicks, using the syndrome as a method of assay. Pappenheimer (176) has described in great detail vitamin E deficiency in adult mice and their offspring. The latter showed necrosis of skeletal muscles and gross edema of the tissues though no lesions were found in the central nervous system. The two muscular dystrophies in rabbits, one due to lack of vitamin E, the other to feeding of fish liver oil, are stated to be identical syndromes (177).

Vitamin E deficiency in man.—There have continued the unfruitful attempts to identify various human conditions, which have come about naturally, with symptoms which are induced in animals by gross distortions of their diet designed to rob them of vitamin E. Although the vitamin remains advocated as a precautionary measure by obstetricians (178) and is said to augment the estrogens (179, 180), it is admitted to be no positive remedy for habitual abortion. Its usefulness in amyotrophic lateral sclerosis, with (181, 182) or without (183, 184) pyridoxin, appears disproved. A comprehensive test (185) of the natural mixed tocopherols, part of the time with thiamin, for progressive muscular hystrophy failed to show cures or even temporary relief in a number of Minneapolis children. As the *Lancet* remarks (186), "Vitamin E Falls from Grace."

Relief of tabes dorsalis (187) has been reported from treatment with vitamins B and E together though the proportion of benefit derived from each vitamin is not clear. In one syndrome only—primary fibrositis—does vitamin E appear to afford indisputable benefit. Here, Steinberg (188, 189, 190) defines a group of symptoms which include aching limbs, fibrillar nodules on the arms and legs near the joints, accompanied often by creatinuria. Following vitamin E therapy for from 8 to 30 weeks, in which γ-tocopherol appears as effective as α-tocopherol (190), the creatine excretion drops dramatically, the nodules shrink, and the muscular distress is relieved. Doses of from 100 to 200 mg. of tocopherols have been employed in the form of wheat germ oil, the synthetic acetate, distilled natural tocopherols, and various pure experimental esters. Success has been uniform except occasionally with wheat germ oil. The advantages, if any, of the intra-

muscular over the oral route and the size of the minimum effective dose have yet to be delineated.

Vitamin E and the control of oxidation.—Ever since the tocopherols were recognized as being responsible for the delay of rancidity (191) of natural oils and fats, there have been attempts to explain (192) their antisterility and antidystrophic activities in terms of oxidation control. It was soon concluded that if this fundamental relationship exists, it is not a direct one. John (193), for instance, stated that there is no obvious connection between the redox potential of tocopherol and a host of related compounds and their ability to prevent sterility in the rat. This failure to establish any connection discouraged for nearly a decade the examination of other ways in which the tocopherols might influence the body in health and disease.

However, during the past two years new experiments have been devised unhampered by the older concepts. The experiments include: measurements of the influence of tocopherols on tissue metabolism; measurements of synergy with other vitamins; and recently, measurements made with radioactive elements[v] (194) on the variation of phosphorus turnover with tocopherol intake.

Tissue metabolism.—Viewing the problem in retrospect, following the suggestions of Szent-Györgyi (195) and others (196), there are a number of ways that tocopherols could influence muscle: (*a*) as a general depressant of metabolism, (*b*) as an inhibitor of spontaneous or involuntary oxidation, (*c*) as a component of the main oxidative cycle of muscle in action, and (*d*) as an essential constituent or building brick required for the formation and maintenance of tissue, the constituent possibly, but not necessarily, being concerned with the main oxidative cycle of muscle activity.

As regards (*a*) the total metabolism of guinea pigs (197) was not found sensibly altered on a low vitamin E diet though that of rats (196) was increased; nor was the total metabolism (198) decreased by a high intake of tocopherols.

An important series of experiments on living and dead muscle has been initiated by Mattill (199, 200) and is still being carried on by his associates (201). Rabbits or rats, deprived of vitamin E until they were in a helpless condition and near death, were killed by a blow on the head. Dystrophic muscle, which proved to be white and insensitive to irritation, was removed and placed in a phosphate saline solution in Warburg flasks at 38° and the oxygen turnover calculated from the carbon dioxide absorbed. Parallel experiments were done

on normal animals and dystrophic animals which had received tocopherol acetate before operation. The dystrophic muscle of young rats had an average oxygen uptake of 3.73 c.mm. per hour per mg. of dry tissue, dystrophic adults averaged 2.52, while normal rats stood lower at 2.19 c.mm. The values for rabbits were 1.6 for normal and 2.18 for depleted. The response to α-tocopherol in these experiments was neither uniform nor large. All measurements quoted are on the first hour in the Warburg flask.

The work has been extended with hamsters (202) as experimental animals with α-tocopherol phosphate as a vitamin E derivative diffusible in water, and with removal of small pieces of muscle from living animals under an anesthetic which does not disturb the fat balance of the organs. With this technique Houchin showed that the oxygen consumption of the dystrophic hamster was 250 per cent above normal, of the rat 100 per cent above, and both were reduced to near normal twenty-seven hours after oral administration of α-tocopherol acetate, the interval evidently being occupied by diffusion of the vitamin from the intestine. When the curative agent was α-tocopheral phosphate administered parenterally, the oxygen consumption fell 34 per cent in the first hour and returned to normal three hours later. The experiments on excised muscle were repeated *in vitro* with the inclusion of heat-killed specimens among the samples. The tissue slices were placed in Ringer's solution in the Warburg flasks either alone or with tocopherol additions and the oxygen consumption measured. The oxygen quotient for the normal hamster was 1.74; dystrophic hamster alone, 2.89; with tocopherol phosphate in solution, 1.86; with tocopherol acetate given forty-eight hours before death, 1.70. Normal tissue was unaffected either by tocopherol phosphate or the emulsified acetate. Dead tissue showed no oxygen uptake and none was promoted by tocopherol phosphate. Minced and homogenized live muscle showed an uptake of 0.68 c.mm. per hour per mg. for normal and 0.64 for dystrophic—low figures which suggest that the intact live cell is responsible for oxygen uptake as a machine rather than as a depot of reducing agents. Further experiments with minced tissue, cytochrome-*c*, and, occasionally, desoxycholic acid, showed that dystrophic muscle conferred a succinoxidase activity of 160 per cent of that given by normal muscle and that the high value was reduced to normal by incorporation of tocopherol.

The sum total of these experiments led Houchin to the belief that α-tocopherol, possibly as phosphate, is an active participant in the

metabolism of skeletal muscle. However, if the oxygen quotient of normal muscle is called A, with the quotient of dystrophic muscle, A + B, the experiments record that tocopherol diminishes B to zero without affecting A. Is there yet enough evidence to decide that it is responsible for A?

Other measurements bear on the situation. Although fat-soluble, the vitamin E in the living animal is not necessarily held in the fatty portion of the body, or even preferentially so. Mason (203), investigating the distribution of vitamin E in the rat, found that the heart and lung, weight for weight, held 1.5 to 2 times as much as the body fat and considerably more than the liver, except when excessive doses were fed. Since the fat content of the heart and lung is only from 10 to 15 per cent of their dry weight, either the fats of these organs contain from five to ten times the concentration of tocopherols held by the rest of the body fat or the tocopherols are combined with or adsorbed to the nonfatty material. Devlin & Mattill (170) devised a method for estimating tocopherols in small samples of tissue and found that tocopherol added *in vitro* to minced tissue could not be recovered quantitatively unless freed by sulfuric acid. Again, Mason found more vitamin E in heart and lung than in liver, the normal storage place for fat-soluble vitamins. This is in contrast to vitamin A which, in ester form, collects preferentially in the liver. Evidently the free hydroxyl group of the usually unesterified tocopherol confers an affinity for tissue; as a descriptive term, we may say that tocopherol "dyes" tissue. In this dyed condition tissue is normal; undyed or leached by diets poor in vitamin E or rich in unsaturated acids which attack the vitamin, the tissue is dystrophic. The quantity of tocopherols involved is very small. The amount required to protect against muscle dystrophy in the rabbit has been measured by Eppstein & Morgulis (204, 205) who find that as little as 0.2 to 0.69 mg. per day per kg. of body weight will protect the rabbit when administered parenterally as the disodium phosphate of natural α-tocopherol. This compares favorably with the 0.7 to 0.8 mg. of *d,l*-α-tocopherol acetate required orally.

Synergistic action of the tocopherols.—The many occasions in which vitamin E has been found to increase the biologic activity or organic storage of other vitamins have been traced during the year to a protective action (191, 206) of vitamin E on fats within the animal body. The tocopherols are ubiquitous; they or alternatives such as gossypol (207) occur wherever fat is handled in nature. The vitamin

appears in all common vegetable seed oils and even in the liver oils of fishes (18). The stability of rendered animal fat is influenced by the tocopherol content (208); that of vegetable oils is related thereto. The tocopherols are typically synergistic substances, seldom exerting their anti-oxidant action alone. The trace of vitamin E (0.04 per cent) which stabilizes corn oil, if extracted and added to a similar quantity of "pure" distilled corn oil, fails to achieve equal stabilization. In the natural oil the tocopherol is accompanied by phospholipids, low molecular weight phenolic bodies (209), free fatty acids, and perhaps other factors which, acting together, confer a stability from 10 to 100 times more than we associate with pure tocopherol and oil. The tocopherol is the primary inhibitor because the other constituents alone confer less stability than tocopherol alone. Golumbic (210) lists three classes of tocopherol promoters: acids such as ascorbic, phosphoric, or pyruvic; phospholipids such as lecithin and cephalin; phenols such as tannins and gallic acid. The synergy between the mixed tocopherols and ascorbic acid (211) is surprising. The acid is substantially insoluble in lard, a few milligrams added to a kilogram apparently failing to dissolve; yet lard so treated becomes phenomenally stable after addition of tocopherol. Typical Swift test induction periods are: refined lard, 1 hour; lard plus tocopherol 0.02 per cent, 20 hours; lard plus ascorbic acid plus tocopherol 0.02 per cent, 300 hours. In the animal organism there are supplies of ascorbic acid, phospholipids, and probably tannins in sufficient quantities for the activation of tocopherols at the site of operation. The quantity of vitamin E in the fat and tissues of the rat [average figures obtained by Mason (203) on usual (low-E?) diet] is one rat m.f.d., say 1 mg. in 50 gm., or 0.002 per cent, which is about one tenth the figure chosen by Taylor & Jakobsen (212) for the optimum costabilization of lard.

In the last few pages we have summarized current evidence for the protective role of the tocopherols on two main body constituents, tissue and fat. It remains to describe the protective action on trace constituents dissolved therein. Of these entities, carotene, vitamin A (19), and perhaps progesterone (179) are already known to receive protection. Since each trace substance has a different inherent stability and a different response to protective agents, and the agents themselves are the result of a complicated synergy, the total relationship, trace substance $\rightleftharpoons$ substrate $\rightleftharpoons$ tocopherol mixture $\rightleftharpoons$ quinone balance $\rightleftharpoons$ tocopherol activators $\rightleftharpoons$ etc., has qualitative and quan-

titative aspects of vast numerical proportions which will require exploring in many mixtures and levels of dosage.

Synergy with vitamin A.—Descriptions of this synergy (19) and methods of experiment (159) have been given already. Fed simultaneously, the tocopherols exert the following enhancements of the A vitamins under the conditions employed: 1 μg. of vitamin A is 1.5 to 2 times more effective in the presence of optimum vitamin E than in its complete absence; 4 μg. of A are noticeably enhanced; the apparent activity of 30 μg. A is not increased. The apparent potency of 1 μg. of carotene is multiplied by 2 to 2.5 of 4 μg. by 1.25 to 1.5. The apparent potency of 40 μg. is not improved by vitamin E. These figures are for young rats weighing 50 to 100 gm. The optimum quantity daily for rats of this size, eating 10 to 12 gm. of food daily, is 0.15 mg. tocopherol for vitamin A, 0.6 mg. for carotene. Transferred to human requirements this becomes 100 and 400 mg. daily on a weight conversion and 9 and 35 mg. on a food intake basis. The latter figures are considered more reasonable and are comparable with the 4 to 8 mg. found in human diets.

The equivalence of the tocopherols (159) in sparing the A vitamins has been examined: Rats fed 0.8 μg. of carotene in the absence of vitamin E showed 2 gm. growth increase in 36 days. With a 0.15 mg. supplement of mixed tocopherols, 31 gm.; pure α-tocopherol, 27 gm.; pure β-tocopherol, 30 gm.; pure γ-tocopherol, 30 gm. With 0.1 mg. supplement of ascorbic acid, 7.5 gm.; with 1 mg., 11 gm.; with 10 mg., 20 gm. With 0.13 mg. of hydroquinone, 3.3 gm.; with 35 mg. lauryl hydroquinone, 27.9 gm., and with 0.5 mg. palmityl ascorbic acid, 30 gm.

The ability of high dosages of ascorbic acid to enhance vitamin A is significant. Whether the protection is exerted at the interface of the fat depots or on the vitamin A which at any moment is circulating in the blood stream is undecided though the latter is suspected. The other substances tested behaved, as would be anticipated, according to their solubility in water and fat.

As to the seat of action, this is evidently the intestine because vitamin E administered on alternate days with the vitamin A showed a much diminished effect compared with administration at the same time. In all the experiments the vitamin supplements were given by medicine dropper. Strictly equivalent effects were not observed when they were mixed with the foods. In fact, in some cases the addition of tocopherols by volatile solvent to the powdered mixed feed

resulted in decrease of vitamin A response. Evidently vitamin E in nature is protected mechanically and chemically in the cell. The application of free vitamin E to commercial feeds is not yet sufficiently developed to duplicate the laboratory experiments.

Synergy with vitamin C.—The ability of ascorbic acid to replace tocopherol in protecting vitamin A in the rat leads to the question of whether the ascorbic acid itself can be protected by vitamin E (159). The accumulation of vitamin C in the organs of guinea pigs was examined on low ascorbic acid intake and variable tocopherol intake. Animals given 6 to 10 mg. ascorbic acid per kg. of body weight and 0.5 to 1.0 mg. additional tocopherol per kg. showed increases of vitamin C in the liver of 19 to 22 per cent; in the adrenals, 22 to 66 per cent. Larger quantities of vitamin E produced a lesser effect.

While there are no positive reports (213) of the interaction of vitamin E with other water-soluble factors, there are the very suggestive experiments of Quackenbush *et al.* (214) showing that unsaturated fats or pure linoleates are interchangeable with mixtures of pyridoxin and pantothenic acid or rice bran extracts in curing rat acrodynia. When the connection between vitamin E and the unsaturated fatty acids in producing dystrophy in herbivora is borne in mind, it is probable that a complicated synergy between the three factors exists.

Antagonism with vitamin D.—Harris has noticed (159) in testing very low levels of vitamin D by the line test that addition of substantial quantities of vitamin E depressed healing of the epiphysis. A qualitative analysis of the depression of antirachitic activity is as follows: with 0.75 units daily of vitamin D_2 or D_3, the healing of the young rats' joints is repressed by 1 mg. of tocopherols daily; unaffected by 0.33 mg. daily. With 1 unit of vitamin D daily, the healing is unaffected by any dose (1 to 9 mg.) of vitamin E. Translated to human therapy in terms of food intake, there should be no depression of minimal quantities of vitamin D with less than 23 mg. tocopherols daily. With 600 units of D daily, rickets would not be induced by any dosage of vitamin E. Weissberger & Harris[9] (194), experimenting on the two vitamins and radioactive phosphorus, have found that the phosphorus turnover of the young rats' bones is greatly increased under conditions of very low and very high tocopherol intake. The effect is not found with moderate supplements.

Synergy with sex hormones.—Minimal quantities of vitamin E are necessary to insure development of the testes in the growing male

and to maintain fertility in the female. It has not been established that larger quantities of tocopherols promote increased sexual activity. Mason lists the known facts (215) with a full bibliography and Bacharach & Chance (216) have stated that α-tocopherol acetate has no estrogenic activity for the corpus luteum hormone. Stähler & Pehl, however (179), have demonstrated that vitamin E affects both female hormones—results established by Bach & Winkler (217). In the case of the estrogenic hormone the effect of vitamin E is slight, but the action of progesterone is greatly intensified, two thirds of the customary dose given with α-tocopherol being more effective than the normal dose administered alone. This has been tested in humans and rabbits. Stähler & Kaiser (180) have demonstrated that a certain phase of the development of the uterine mucosa can be obtained with one tenth of the usual dose of progesterone in the presence of adequate amounts of vitamin E.

Passing to the androgen, we notice in a paper by Adamstone (218) a photograph of a castrated cockerel maintained on a high vitamin E diet which has shown incomplete depletion of androgen in the usual sixty-day period of preparation. He finds that under high vitamin E intake cockerels require less testosterone than in the absence of E.

The activity in sparing hormones, if substantiated, would appear to be due less to increased production than to antioxidant protection; thus, the quantity available equals the quantity being continuously produced, minus the quantity being continuously destroyed. Diminish the latter, and the quantity available becomes increased.

Vitamin K

Antihemorrhagic substances have been reviewed in 1942 by Mariani (219), and by Dam (220) from whose paper and letters these paragraphs are largely drawn.

The search for new and more soluble agents continues. The sulfonated (221) derivatives of 2-methyl-naphthalene have been investigated, the potassium bisulfite addition product of 2-methyl-1,4-naphthoquinone being strongly active while the isomeric 2-methyl-1,4-naphthohydroquinone-3-potassium sulfonate had only one tenth the potency. The sodium salt of 1-acetoxy-2-methyl-4-naphthyl-hydrogen succinate was soluble and highly active but was too unstable in solution to be useful (222, 223). The sodium salt of 1-acetoxy-2-methyl-4-naphthyl hydrogen sulfate and of the corresponding phos-

phate were less active but were stable in solution and suitable for either intravenous or intramuscular injection (223). Satisfactory solubility and activity were exhibited by 1-hydroxy-2-methyl-4-naphthyl-β-monoglucoside and β-monomaltoside (223) although the solutions were slowly oxidized by air.

Vitamin K has been prepared in commercial quantities by extracting ground alfalfa with oil (224). A new source (225) of the natural vitamin was found in the berries of the European mountain ash (*Sorbus aucuparia*), extracts from which have been used clinically.

The analysis of vitamin K and analogues has been advanced by Scudi & Buhs (169) who have devised a general method for reducing substances which has already been adapted to vitamin E (168) and members of the B complex (226). The butanol extract of the substance under test is reduced by hydrogen over Raney's nickel and the solution oxidized with 2,6-dichlorphenol indophenol. The prevention of interference by substances which rob the method of specificity has also been discussed (227). In other colorimetric assays a green solution (228) is produced when sodium bisulfite and 2,4-dinitrophenylhydrazine react with vitamin K; a blue solution (229) when the vitamin and derivatives react with sodium pentacyanoammineferroate.

Much attention has been given to the prothrombin time test devised by A. J. Quick (230, 231). This is a rapid one-stage method in which a large excess of thromboplastin is added to the plasma under test. Attempts to further accelerate or render the manipulation more convenient have not always taken into account the factors which influence accuracy. A better study of this kind has been made by Larsen & Plum (232) who have measured the influence of temperature, pH, and method of preparing the thromboplastin on the sensitivity of the test. Instead of using dried commercial preparations made from rabbit brain, they used human thromboplastin that had been neither dried nor treated with acetone. Clinical determination of low prothrombin plasmas was much improved.

The physiological mechanism (233) of prothrombin formation by vitamin K and its inhibition by the hemorrhagic substance in sweet clover disease continue to occupy attention. [There are related studies on heparin (234), on proteolytic enzymes from plasma (235), and on thromboplastin (236) from ox lung.] Rats fed a diet artificially low in vitamin K were seriously affected (237) by small doses of 3,3′-methylene*bis* (4-hydroxy coumarin) which produced marked hypoprothrombinemia, resulting in bleeding wherever tissue was sub-

jected to the slightest trauma. Normal stock diet or one reinforced with vitamin K protected the rats largely though not completely from the coumarin compound. Hypoprothrombinemia has been induced in rats by feeding sulfaguanidine as part of a simplified diet (238). The rat is known to derive vitamin K from intestinal flora, the growth of which is repressed by the sulfa-drug. The effect is counteracted by *p*-aminobenzoic acid or liver extract.

By far the greatest interest clinically has centered around the vitamin K requirements of mother and child just before and after birth. *Nutrition Reviews* devotes two articles (239, 240) to this theme and Dam (220) quotes many references. It has been well established (241, 242) that the prothrombin level of the infant is subnormal at birth and becomes lower during the first three days of extra-uterine life. It has not been completely cleared up which factors play the most important role in the lowering of the prothrombin immediately after birth. The small amount of milk consumed during the first days and the low vitamin K content of the milk (243) may play a role. Vitamin K could not be found in the feces from newborn breast-fed children during the first few days, whereas the feces of artificially fed infants contained more vitamin K than was ingested (244). There is no doubt that part of the mortality among the newborn is attributable to deficiency in vitamin K. At least 20 per cent of the 128,000 infants who fail to survive each year in the United States show evidence of cerebral hemorrhage. A further proportion of infants who are aborted before coming to term are probably lost through lack of the vitamin. The experimental conditions for abortion due to deficiency-K have been established for the rabbit (245).

The treatment of pregnant mothers with vitamin K has been given extended clinical test (246). Hellman & Shettles (247) report that administration of 2 mg. of 2-methyl-1,4-naphthoquinone a few hours before labor diminishes infant mortality from forty to twenty per thousand deliveries. Retinal hemorrhage which can be detected in about 25 per cent of newborn American babies is found in 15 per cent when vitamin K is given during labor, in only 6 per cent when given four days previously. Parks & Sweet (248), using larger doses of K (5 mg.), confirmed that the prothrombin level of the mother's blood was raised but found no decrease of abnormal bleeding in the offspring. Similar experiences (249) have been reported and again vigorously denied (250). The score to date, as *Nutrition Reviews* remarks, is two to one in favor of prelabor medication with vitamin K,

a score which coincides with the known vitamin K deficiency of the newborn.

The fact that 2 mg. of vitamin K, given to a mother and child weighing about 150 lbs., can confer hemorrhagic immunity to the 6 to 8 lb. child a few hours later suggests that the requirements of the infant are small. These have been established as about 1 μg. of derivatives (251) and relatives of 2-methyl-1,4-naphthoquinone (252).

The role of vitamin K in jaundice (253) and tuberculosis has been studied. The jaundiced liver fails to supply bile to the intestine, absorption of the vitamin from the diet is prevented, and the prothrombin level of the blood falls. After the injection of synthetic vitamin K there is a prompt return to normal. The vitamin has been reported to have a similar beneficial influence when given with morphine in cases of pulmonary hemorrhage due to tuberculosis (254).

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NUTRITION, 1941 AND 1942

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This chapter aims not to duplicate what is included in other chapters on the chemistry and metabolism of the nutrient elements and substances but rather to review the advances in our knowledge of the quantitative requirements of human nutrition and the adequacy of typical dietaries or food supplies to meet these nutritional needs.

Early in 1941, the Committee on Food and Nutrition of the National Research Council reached agreement upon, and released for publication, a table of "Recommended Daily Allowances for Specific Nutrients," popularly called the "new yardstick for good nutrition," and recently republished by Wilder & Keys (1) with comments upon its possible international significance. They point out that Sir John Boyd Orr, calling it the "Washington nutrition yardstick," advocates its official recognition as a guide to right relations among the United Nations in the use of their total food supplies.

Eight of the columns of "Recommended Allowances" are here reproduced as Table I, which will serve as a point of departure for this review. The two specific nutrients included in the original table but omitted here are: vitamin D, because it is so largely a separate story; and nicotinic acid, because of doubt whether the data yet available are such as to permit of comparisons at once comprehensive and quantitatively valid between amounts required in nutrition and amounts furnished by food supplies.

Energy, Protein, and Iron

Publications of the past two years confirm the view that the "Recommended Allowances of the National Research Council" provide ample margins above the actual needs of normal people for energy, protein, and iron; and also that American dietaries, when meeting these standards of energy value, only rarely fall below the needed levels of protein and iron.

Pittman, McKay, Kunerth, and co-workers (2) conclude from their studies of the dietaries of twenty-seven normal women (twelve living in Kansas and fifteen in Ohio) that, while the "Recommended

TABLE I

Recommended Daily Allowances for Specific Nutrients*

(Committee on Foods and Nutrition, National Research Council)

	Calories	Protein gm.	Calcium gm.	Iron mg.	Vitamin A† I.U.	Thiamin (B_1) mg.‡	Riboflavin mg.	Ascorbic acid mg.‡
Man (70 Kg.)								
Moderately active	3000	70	0.8	12	5000	1.8	2.7	75
Very active.....	4500					2.3	3.3	
Sedentary	2500					1.5	2.2	
Woman (56 Kg.)								
Moderately active	2500	60	0.8	12	5000	1.5	2.2	70
Very active.....	3000					1.8	2.7	
Sedentary	2100					1.2	1.8	
Pregnancy (latter half).....	2500	85	1.5	15	6000	1.8	2.5	100
Lactation.....	3000	100	2.0	15	8000	2.3	3.0	150
Children up to 12 years:								
Under 1 year§.....	100/Kg.	3–4/Kg.	1.0	6	1500	0.4	0.6	30
1–3 years**.....	1200	40	1.0	7	2000	0.6	0.9	35
4–6 years.....	1600	50	1.0	8	2500	0.8	1.2	50
7–9 years.....	2000	60	1.0	10	3500	1.0	1.5	60
10–12 years.....	2500	70	1.0	12	4500	1.2	1.8	75
Children over 12 years:								
Girls, 13–15 years	2800	80	1.3	15	5000	1.4	2.0	80
16–20 years	2400	75	1.0	15	5000	1.2	1.8	80
Boys, 13–15 years.	3200	85	1.4	15	5000	1.6	2.4	90
16–20 years.	3800	100	1.4	15	6000	2.0	3.0	100

* Tentative goal toward which to aim in planning practical dietaries; can be met by a good diet of natural foods. Such a diet will also provide other minerals and vitamins, the requirements for which are less well known.

† Requirements may be less it provided as vitamin A; greater if provided chiefly as the pro-vitamin carotene.

‡ 1 mg. thiamin equals 333 I.U.; 1 mg. ascorbic acid equals 20 I.U.

§ Needs of infants increase from month to month. The amounts given are for approximately 6–8 months. The amounts of protein and calcium needed are less if derived from breast milk.

** Allowances are based on needs for the middle year in each group (as 2, 5, 8, etc.), and for moderate activity.

Allowances" provide 2500 Calories[1] a day for a moderately active woman, the "previous customary dietary standard" of 2400 Calories is comfortably above their average finding of 2038 Calories of *ad libitum* food consumption. Their individual data varied, however, from 1119 to 3267 Calories. With a variation so wide as this, it may be questioned whether twenty-seven cases are sufficient to establish a stable general average.

The allowance of approximately 1 gm. of food protein per day per kg. of body weight for normal adult maintenance is stated by Lewis (3) to provide a margin of 50 to 100 per cent above actual need. This fact is, perhaps, too often forgotten by those whose attention is strongly attracted to medical studies of the protein-deficiency state and to the fear of recurrence under war conditions of the high incidence of "war edema" reported during the aftermath of the First World War and supposed to have been due in large measure to subsistence upon protein-poor food. Youmans' recent studies emphasize (4, p. 231) the fact that protein deficiency of dietary origin is seldom if ever found except where there has been shortage of total food calories as well as a diet of low-protein content. And again Youmans writes (4, pp. 237–38),

> The total caloric intake has an exceedingly important bearing on the adequacy of the protein supply. With a high calorie diet the protein intake may be reduced safely to a very low level.

With the very large reserves of wheat which are available, at least to the Americas and Great Britain, the consumption of total food calories can easily be maintained at a sufficiently liberal level to afford protection from danger of protein deficiency even if the hitherto customary per capita consumption of high-protein foods in the United States should be somewhat reduced by sharing with the other United Nations. It may be well, however, for those biochemists who have occasion to deal with such war-emergency questions of nutrition to keep steadily before their public the fact that the less highly refined mill products of wheat (and doubtless also of other grains) contain not only more protein but also protein of higher nutritional efficiency than that of white flour.

This nutritional superiority of the protein mixture of wheat products which approximate the whole grain over that of those which contain the interior of the endosperm only had been shown in growth

[1] In this chapter where Calorie is a definite quantitative measure (and only in such cases) we use the capital C to indicate the kilogram-calorie according to the convention of the physicists.

experiments with rats; it has now been demonstrated directly upon human subjects by Murlin, Marshall & Kochakian (5). As measured by their methods and compared with the "biological value" of egg protein as 100, the proteins of four approximately whole wheat breads showed 81.6, 77.8, 79.6, and 81.0, respectively. Unimproved white bread protein showed a corresponding value of 74.6. Fortification of this bread with a complete B vitamin concentrate resulted in a protein efficiency value of 75.3 while the protein mixture of white bread "containing 5 per cent non-fat milk solids," baked with high vitamin yeast, and "eaten with no extra B vitamins" showed an efficiency of 79.8.

Both with respect to protein and to iron, there is a tendency which biochemists are perhaps best qualified to evaluate and correct and which needs correction if the nutritional needs of the population are to be rightly understood. We refer to the tendency to be too much influenced in our estimate of normal needs by the small proportion of idiosyncratic people whose needs are abnormally high because the efficiencies of their bodily processes of nutrition are abnormally low in some respect. Such cases are, of course, important medical responsibilities and justify ample discussion in medical meetings and literature. But one should not infer that there is new evidence favoring higher protein intakes for normal people.

Another influence tending to overestimates of both protein and iron requirements is the inclusion of the data of balance experiments in which the intakes were not sufficiently low to "put the body to the test" nor sufficiently uniform to permit of valid interpretation. Experiments with higher or less constant intakes may throw valuable light upon other biochemical problems, but the inclusion of their data has undoubtedly contributed to overestimates of the normal need; such overestimates are to some extent still current and perhaps unduly retard the application of more critical evidence.

Confining attention to the investigations which now seem well suited to afford fully valid evidence on the question of the amounts of iron actually needed for normal nutrition, the work of the past two years seems clearly to show that the need is lower than previously supposed and, therefore, that the 1941 "Recommended Allowances" carries even more liberal margins ("for safety" or to cover individual physiological variations) than appeared at the time of its adoption.

Leverton (6) reports rigorously planned and controlled iron-balance experiments at two levels of intake low enough to yield valid

evidence as to real requirement; as in the similarly planned work of Farrar & Goldhamer (6a) and of Vahlteich, Funnell, MacLeod & Rose (6b), she finds very much less iron needed for normal maintenance than is suggested by the "Recommended Allowances of the National Research Council Committee." In a comprehensive series of balance experiments using a mixed diet of ordinary food materials of low iron content, Leverton's subjects while receiving 3.5 mg. of iron per normal adult per day excreted an average of 3.83 mg. With the intake raised to 6.55 mg. the average output rose only to 4.31 mg. Apparently the average maintenance requirement of these healthy adults must have been very close to 4 mg. per day. Farrar & Goldhamer had concluded that the experiments which up to the time of their publication had been best planned and controlled indicated a maintenance requirement of about 5 mg.; the four cases reported by Vahlteich, Funnell, MacLeod & Rose, in which intakes were not so rigorously restricted, yielded figures of only 6.2, 5.7, 6.1, and 6.1 mg. per day, respectively.

The view long held by some that other constituents of the diet may be extremely important in determining the efficiency with which its iron is utilized by the body has been emphasized anew by Leverton & Marsh (7). They found, for example, that seven young women whose self-chosen diets provided an average of 7.21 mg. of iron daily and were, according to the authors, "superior" in other respects stored 1.55 mg. of iron daily; five others who chose diets of the same iron content (7.09 mg.) but less favorable as to other constituents showed a net loss of 1.30 mg. of iron daily. The intake of calcium by the former group was 58 per cent higher, of phosphorus, 35 per cent higher, and of protein, 34 per cent higher than by the latter group. Other factors, doubtless, also shared in the superiority of the former diets.

Thus if the best evidence now available be regarded unmixed with the data formerly accepted but which now appear less valid, one may well agree with Leverton's suggestion

> that the amount of iron may not warrant special consideration in dietary practices or recommendations but that an ordinary mixed diet adequate in the other known essentials would supply ample iron for (normal) maintenance and replacement. . . . .

The literature of requirements for iron has recently been reviewed by Heath (8). In some respects his discussion emphasizes the confusion between the various contributions which have been offered as

evidence; yet it leaves one with the distinct impression that so-called iron deficiencies (yielding readily to therapeutic doses of iron salts) are more often due to extra-physiological demands arising within the body than to faulty food; that they belong, therefore, rather to the responsibilities of medical practice than of normal nutrition; and that the nutritionist's responsibility will be met, without any separate anxiety as to iron, by the successful teaching of such dietaries as a modern nutritionist would teach in any case.

Correspondingly, the degree of emphasis to place upon hemoglobin levels in assessing the nutritional status remains a problem. On the basis of a special analysis of the values observed in a high-income, well-nourished group of high school students in New York City, Wiehl & Kruse (9, 10) have offered tentative standards which take account of both chronological and physiological age. By these standards, none of the boys and only 3.1 per cent of the girls of the high-income group were anemic. The incidence of anemia was slightly higher (boys 2.5, girls 4.3 per cent) among a low-income group of high school students, but still very low in comparison both with the frequency of iron intakes far below "recommendations" and with the prevalence of objective signs of other nutritional failings (discussed below). Similarly, in a group of New York City W.P.A. workers 17 to 65 years old, the frequency of hemoglobin values below 14.0 gm. per 100 ml. for the men (1.3 per cent) and below 12.0 gm. per 100 ml. for the women (6.8 per cent) was much lower than that of objective evidences of avitaminosis A, ariboflavinosis, and subnormal intakes of vitamin C.

On the other hand, in a mass study in Pennsylvania (11), low hemoglobin values were much more frequently observed. The survey included 100 urban and 100 rural families, plus 1492 additional grade school children—a total of 2000 individuals. Of the men, 12.5 per cent of those aged 17 to 20, 34 per cent of those aged 20 to 40, and 37 per cent of those over 40 years had hemoglobin values below 13 gm. per 100 ml. Among the women of the corresponding age groups, the incidence of hemoglobin values below 11.5 gm. per 100 ml. was 13.1 per cent, 36 per cent, and 27 per cent, respectively. The average hemoglobin value was distinctly higher for rural than for urban men over 20. The range of values at all ages was much greater in the city than in the farm families, possibly because "the rural diets showed far less variety in type than did those of the urban families." Though differences in the methods of reporting the data make impossible an exact comparison of the Pennsylvania and the New York City children of

high school age, it seems that relatively low hemoglobin values were far more common among the former: e.g., 26 per cent of the Pennsylvania girls 12 to 17 years old had values below 11.5 gm. of hemoglobin per 100 ml.

Calcium

Chu and co-workers (12), from what seems to have been a very significant study of the calcium requirement of maintenance, report an average finding of 0.0073 gm. of calcium per kg. of body weight per day; six healthy men and six healthy women served as the experimental subjects. On the usual basis of comparison, this corresponds to 0.51 gm. for the "average man" (of 70 kg.) as compared with an average of 0.45 indicated by a compilation of evidence available in 1920 and the finding of 0.55 gm. by Leitch in 1937–38 for adults, regardless of body weight.

Simultaneously, two investigations at the University of Illinois have yielded higher results which, however, in our judgment are not necessarily inconsistent with the other evidence. Outhouse and co-workers (13) find with seven subjects an average of 0.75 gm., and Steggerda & Mitchell (14) with nine subjects an average of 0.67 gm. per 70 kg. of body weight for the full support of calcium equilibrium. The explanation given by Mitchell and by Outhouse for their higher findings is undoubtedly correct in its main purport, though some details of the discussion which the Illinois workers offer in explaining their correction of earlier work might in turn be correctively clarified in a fuller discussion.

For this brief review, let it suffice to say that the findings of Outhouse and of Mitchell, with their respective co-workers, are reflected in full by the Federal Food Administration's definition of "minimum" calcium requirement for adults as 0.75 gm. per day, while the National Research Council's Committee gives ultra-conservative recognition in its "Recommended Allowances" of 0.8 gm. for adult maintenance. This committee, however, recommended 1.5 gm. for women in the latter half of pregnancy and 2.0 gm. during lactation, with 1.0 gm. for infants and 1.0 to 1.4 gm. for children and adolescents. Still more recently workers in six state institutions have published a joint contribution on calcium, phosphorus, and nitrogen metabolism of college women (15). With authorship so widely divided, the present reviewers trust they may suggest, without offense, that an apparent self-contradiction between certain parts of this important paper will be avoided if we may read "indicated" in some of the cases in which the

word "showed" or "shown" appears. Earlier work had indicated that calcium requirements as computed from the data of balance experiments with individuals on fixed diets may not be universally applicable to people on different and varying diets. The paper now reviewed (15) confirms and extends the general view just cited and presents differences which in the opinion of its authors

> seem large enough to warrant the conclusion that the nature of the diet was a factor in calcium retentions and that the controlled diets had a stabilizing effect upon such retentions.

Thus the investigators were led to include in their work 124 balance experiments with college women "on their customary self-chosen diets." Of the data of these experiments the investigators write:

> Results showed that place, age, and weight differences were not significant for the 124 subjects, and that intake was significantly related to retention. Figures of regression of balance upon intake showed that mean daily intakes of 0.8 gm. calcium, 1 gm. phosphorus, and 9 gm. nitrogen, respectively, were required for equilibrium.

If this literally shows the mean calcium need of such women, then the "Recommended Daily Allowances" of the Committee on Food and Nutrition of the National Research Council provides no margin above the mean of absolute need. Even if we call this indicated rather than shown, it strongly supports the view that the allowance of 0.8 gm. of calcium for adult maintenance might better be increased to 1.0 gm.

Meanwhile animal experimentation has yielded a large amount of evidence, some as yet unpublished and some published only in brief, that liberal calcium allowances are good for adults of both sexes and at all times—not merely for pregnant and lactating mothers. The effects of different liberal levels of calcium intake have been studied through the entire life histories of hundreds of rats, and in a large proportion of these cases, through successive generations. Other hundreds of strictly parallel animals have been killed at fixed ages for quantitative determination of body calcium (16, 17, 18, 19). In barest outline the chief findings of the full-life and successive-generation experiments just mentioned were as follows: When the calcium content of a diet which originally contained an amount slightly above that of minimal adequacy[2] was approximately doubled, there resulted

[2] This was *"Diet A"* (Laboratory No. 16) containing 0.19 to 0.20 per cent of calcium in the air-dry food mixture whose energy value is approximately 4.0 Calories per gm. Rat families in the laboratory of the Columbia Department of Chemistry are still thriving on this diet in the fifty-fourth generation.

better development, a higher level and longer period of adult capacity and vitality, and longer life without increased incidence of calcification of arteries but with a better store of calcium in the skeletal system. When the percentage of calcium in the food was increased only to 0.34 per cent of the dry solids (or about 70 per cent above the original calcium level), the increased adult life-expectation was well marked for the males and only negligible for the corresponding females, which latter, however, had reared many more young at the higher level of calcium intake. When all females were kept unmated, those receiving the higher of these two levels of calcium intake lived longer than those on the level but slightly above minimal adequacy. Moreover, at still higher levels of calcium intake females both reared more young and lived longer.

Scientific probability strongly supports the view that these general relationships of calcium intake to nutritional well-being throughout the life span are equally true for the human species. A liberal margin of calcium intake above the literal requirement of maintenance thus appears clearly desirable for all; but a direct measurement of the lifetime optimum with human subjects will doubtless present difficulties and require a long time. Meanwhile we believe it will be a worthwhile step in the direction of optimal human nutrition to increase the recommended daily allowance for adults at maintenance from the 0.8 gm. of Table I to perhaps 1.0 gm. per day.

Nutritional availability of the calcium of foods.—Fincke (20) finds the calcium of broccoli and of cauliflower to be comparable in nutritional efficiency with that of kale, these three foods being but little below milk in the efficiency of utilization of their calcium. Outhouse and co-workers (21), using a different method, have studied the utilization of the calcium of carrots by seven human adults with highly variable results, from the trend of which it would appear that the calcium of the carrot, while much more available than that of spinach, is probably somewhat less available than that of broccoli, cabbage, cauliflower, lettuce, or kale.

Vitamin A

The question, how much is required, may receive widely varying answers in the case of vitamin A, depending on the criteria of adequacy. Studies several years ago by the United States Bureau of Home Economics (22, 23) were designed to determine the amount of vitamin A required to maintain normal dark adaptation in adults.

There are, however, numerous recent indications that substantially larger amounts may be required to satisfy other criteria. Thus it was found with young rats by Lewis, Bodansky, Falk & McGuire (24) that "to prevent gross and histological evidence of vitamin A deficiency" 2 I.U. daily (approximately 20 units per kg. of body weight) were required; this intake, in most cases, supported maximal concentrations of vitamin A in the retina and made it "probable that the animals in this group would have had normal vision in the dark"; but the intake necessary to support the most rapid growth was five to twelve and one half times as great, and that to permit maximal levels of vitamin A in the blood was twelve and one half to twenty-five times as great, while still higher intakes further increased the liver stores. Observations on infants indicated (24, 25, 26, 27) a similar wide ratio of intake required for maximum blood levels to intake required for normal dark adaptation and suggested further that "the level of vitamin A in the blood may be a more sensitive indicator of vitamin A deficiency than is the result of the dark adaptation test" (27). Correspondingly, when experimental animals were placed on vitamin A free diets,

> the plasma vitamin A concentration remained high in the animals with large liver stores, but fell rapidly in those with low reserves. The retinal concentration of vitamin A, however, remained high despite the low vitamin A plasma concentration and the absence of vitamin A in the liver.

That the retinal concentration of vitamin A may remain high after the liver stores of this factor have been exhausted was noted also by Brenner, Brookes & Roberts (28). In their experience, however, the blood level likewise appeared an unreliable criterion of vitamin A deficiency (depletion of liver stores). Ralli and co-workers (29) correlate several pathological conditions—whether as cause or effect—with depletion of vitamin A in the human liver; their finding of over 1200 I.U. in each gram of normal liver and but less than half that concentration in cardiovascular cases illustrates the magnitude of the replacement process that may be called for in persons whose bodily stores of vitamin A have become depleted from whatever cause.

Procedures for measuring the dark adaptation as an indication of nutritional status continue to be tested and used (30, 31, 32, 33, 34). In the Milbank series, Hunt (30) found that a high-income group of 136 children 12 to 19 years of age had a lower mean visual threshold than 131 of the same ages from a low-income group (2.540 log units as compared with 2.765 log units after 20 minutes, measured with

the Hecht adaptometer under specified test conditions). A survey of food intake indicated that the diets of the former tended to be much more nearly adequate than those of the latter, the greatest difference seeming to be in their vitamin A value. However, against the presumption that this alone might account for the difference in adaptation, Hunt calls attention to the fact that the diets of the two groups show large differences also in vitamin C content and considerable differences in the adequacy of energy intake (as well as of other factors). She points out that both vitamin C (36) and hypoglycemia (37) have been reported to influence dark adaptation.

That a single measurement of visual threshold should not be relied upon to indicate the vitamin A status of an individual was stressed by Yarbrough & Dann (34). They found "normal" adaptations in 151 out of 154 persons in a North Carolina rural mill village, notwithstanding the fact that plasma levels of carotene (131.2 ± 59.4 µg. per 100 ml.) and of vitamin A (51 ± 17.2 I.U. per 100 ml.) as well as reported dietaries suggested that the community was in a condition of subnormal nutrition with respect to vitamin A. They concur in the view of Steven & Wald (31) that "a more searching criterion than low or high threshold is needed to estimate vitamin A deficiency" and they feel also necessary to test the vitamin A lability of the threshold, i.e., the lowering of the threshold after treatment of the subject with vitamin A. Even this criterion is called into question by Hunt & Hayden (35) who followed the visual thresholds (at 33.5 minutes) in a low-income group (of whom a large proportion had xerosis conjunctivae) before and during vitamin A therapy, and in a similar group given no treatment. Of both the treated and untreated groups about two thirds of the subjects (though their thresholds varied from day to day) showed no particular upward or downward trend in dark adaptation in the course of the observations, while of those showing consistent change in threshold, half in each group had increasing, and half diminishing, visual threshold values.

Follicular conjunctivitis, apparently a manifestation of vitamin A deficiency, was found by Sandels and co-workers (38) in 22 per cent of 1041 Florida school children. The condition responded to vitamin A therapy, but complete eradication of symptoms required several months in some cases. From the dietaries reported by 42 children with and by 16 children without follicular conjunctivitis, it was estimated that three fourths of the former but less than a fifth of the latter had vitamin A intakes below 100 U.S.P. units per kg. of body

weight. All but one of the remaining children with follicular conjunctivitis and about a third of the "controls" had intakes of 100 to 160 U.S.P. units per kg., while intakes above 160 units per kg. were associated with freedom from follicular conjunctivitis in all but one case, where practically the entire vitamin A value was derived from sweet potatoes. Correspondingly, it was suggested that when carotene supplies a large part of the vitamin A value of the diet, children 6 to 12 years old should be provided with at least 200 to 250 U.S.P. units per kg.

Kruse (39) reports that xerosis conjunctivae, the first stages of which he detects by biomicroscopy, is an earlier indication than night blindness of vitamin A deficiency in the groups which he observed; the incidence of vitamin A subnutrition which he finds by this criterion far exceeds estimates based on dark adaptation tests. His experience lends support to the higher recommendations for human intake of vitamin A which have become current in recent years.

Thiamin

Wilder and co-workers have continued their experimental studies of the "subclinical" thiamin deficiency state and the amount of the vitamin required for its complete prevention. In their paper most recently received at the time of the present writing (40), they review briefly their earlier work. In this, symptoms which they learned to distinguish as due to shortage of thiamin were induced by a diet almost devoid of the vitamin while none of the signs appeared in any of the controls whose total thiamin intake was 1.1 mg. per day. As these observers were exceptionally qualified to recognize the slightest sign, it is apparent that 1.1 mg. per day met the requirement, not only of the average, but of the individual who required most. In their later experiments they used a basal diet made up of staple (but, of course, low-thiamin) foods which furnished 0.22 mg. thiamin per 1000 Calories or an average of 0.45 mg. per capita per day. The subjects were eleven women between 23 and 46 years old. On the daily intake of 0.45 mg. thiamin, which is characterized as

> an amount not much if any below that provided by the "poor" diets reported in the Stiebeling-Phipard survey of purchases of food by American families

symptoms appeared in from 89 to 196 days. After 132 days, six of the women served for studies of requirement while five continued on the 0.45 mg. level for further study of the symptoms. These are described as

marked changes of attitude, diminished inclination to perform accustomed tasks and progressive decrease of ability to make social adjustments within the group . . . . All subjects became irritable, depressed, quarrelsome, uncooperative . . . . All became inefficient in their work. In part this could be attributed to weakness, in part to inability to concentrate, confusion of thoughts and uncertainty of memory . . . . All subjects lost manual dexterity.

The investigators add:

It should be emphasized repeatedly that these abnormalities of behavior and efficiency had not been present during the period of preliminary observation, that they developed only after several weeks of restriction of thiamin and that they disappeared during a subsequent period in which administration of thiamin, without the knowledge of the subjects, was the only modification of the experimental regime.

As in the thiamin-deficient subjects studied by Bueding *et al.* (41) there was a tendency toward high blood pyruvic acid after administration of glucose.

In all cases of induced deficiency of thiamin, mental and physical inefficiency preceded, by weeks or months, other more objective manifestations of deficiency of thiamin.

With stepwise increase of allowance,

the first significant increase in excretion occurred when later the intake of thiamin was increased from 0.4 to 0.5 mg. per 1000 Calories.

While the authors call this an indication of "minimal" requirement, it would seem to be more nearly the requirement for approximate "saturation." The observers considered that

less than 0.5 to 0.6 mg. (per 1000 Calories) did not suffice to prevent some degree of fatigue, irritability, poor appetite, insomnia, soreness of muscles, decreased activity, and a general feeling of "poor health," and on the basis of this information the desirable minimal level of intake of thiamin could be placed at 0.5 to 0.6 mg. per 1000 Calories . . . . benefit was not observed to have been derived in any case from amounts of thiamin greater than 1.0 mg. per 1000 Calories of the diet.

They conclude that "the 'optimal' intake was not less than 0.5 mg. nor more than 1.0 mg. per 1000 Calories" of a diet containing carbohydrates and fats in proportions customary in America.

Melnick (42), who used as criteria of adequacy of intake the thiamin clearance tests of Melnick & Field (43), concludes that the thiamin requirement of the adult is 0.35 mg. per 1000 Calories of food and recommends an intake of 0.5 mg. per 1000 Calories. His criteria include not only the "thiamin balance" but also the mainte-

nance of pyruvate at such a minimum as is found (44) to constitute a most delicate test.

It might be said that 0.5 mg. per 1000 Calories, recommended both by Wilder's group and by Melnick, may be taken as representing a consensus of present opinion as to fully adequate intake of thiamin. According to Williams and co-workers (45), just about that much thiamin will be furnished by the average American diet if it does not undergo other material changes from the food habits of 1935–39 while the enrichment of white bread and flour becomes practically universal. In their re-evaluation of the thiamin content of the average American dietary previous to the inauguration of the enrichment program, these investigators arrived at a substantially lower figure than had most earlier writers. In so doing they based their estimate of the amounts and kinds of foods consumed principally upon the data of Stiebeling & Phipard and other Federal statistics but made new determinations of the thiamin content of individual foods as purchased and as actually consumed. They compute that:

> The thiamin content of the average American diet, such as was consumed by the middle two thirds to three fourths of the population prior to the advent of enriched bread and flour, is about 0.8 mg. per 2500 Calories.

The lowness of this figure as compared with earlier estimates is a reflection of the lower values which they assign (principally on the basis of thiochrome determinations) to most fruits, vegetables, and meats other than pork, especially after cooking.

Riboflavin

Using the above-mentioned estimates of Lane, Johnson & Williams (45) for average American food consumption, tentative findings indicate a mean per capita intake of about 1.8 mg. of riboflavin per day (46). Either at this level or with the maximal increase that could be anticipated from the bread-enrichment program, the estimated mean intake of riboflavin by the people of the United States will still fall short of the "Recommended Daily Allowances" of the Committee on Food and Nutrition of the National Research Council. More precise quantitative evidence upon this requirement in human nutrition is therefore an urgent need. Such studies are reported to be in progress.

Ascorbic Acid

The ascorbic acid requirements of children, and the practical problem of the amounts that various foods contribute as usually eaten,

have been studied by Bessey & White (47). They determined the blood plasma ascorbic acid levels of ninety-three healthy children living in Boston and plotted these data against the intakes as derived from dietary histories which they considered unusually reliable. The age range of these children was 5 to 13 years, and about equal numbers of boys and girls were included. Each child was medically examined on the day of the test and none with evidence of colds or other acute infection was included. The plasma ascorbic acid rose with the intake, as estimated from the consumption of citrus fruits and tomatoes, to a concentration of 1.0 mg. per 100 ml. at an intake from these sources of 45 mg. per day and showed no further change at higher intakes. When an attempt was made, on the other hand, to compute (using food value tables) the total ascorbic acid intake from the entire diet, "no correlation was apparent between the dietary evaluation and the blood plasma values." It thus appeared to the investigators that the ascorbic acid of the food as consumed came so largely from citrus fruits and tomatoes that the contributions of other foods, as eaten, might be regarded as practically negligible. We meet this same view among those who have to do with the practical feeding of the Army; but, also, there are researches in progress directed toward the better conservation of this vitamin in storage and cooking, so that other foods may be made to yield a better contribution to the ascorbic acid intake. [See, for instance, the study of onions by Mayfield & Richardson (48) and by Murphy (49).] Roberts & Roberts (50), who studied intensively the ascorbic acid "requirements" of five children of the same age range as Bessey & White's group, found by direct analysis that their basal diet, free from citrus fruits and tomatoes, provided significant quantities of vitamin C. Although the amounts of supplementary ascorbic acid which they found to be required for "saturation" agreed fairly well with Bessey & White's figure, their estimate of the total intake needed to insure "saturation" was higher: viz., 55 to 75 mg.

Storvick & Hauck (51) have studied further the urinary excretion and plasma concentration of ascorbic acid in relation to the level of intake and the problem of tissue saturation. Their subjects were normal adults, four women and two men, all "prepared" by "saturation of the tissues with vitamin C during a three-day pre-experimental period." Each experiment to test the effects of a given level of intake lasted 28 days, of which the first two were considered as "for adjustment" of the body, and the last one was used in testing bodily condition by means of a large test dose of ascorbic acid. The

intervening 25 days were averaged. All six of the subjects were thus studied at the intake level of 200 mg. of the vitamin per day; and from one to three of them at each of eight lower levels: 150, 125, 100, 90, 75, 65, 55, and 50 mg., respectively. At the intakes of 75 to 50 mg. the experiments were less numerous, and the individual differerences were pronounced. One subject still showed "saturation" at an intake of 65 mg., while another dropped from a test dose output of 207 to one of 76 mg. when the intake had been reduced from the 100 mg. to the 75 mg. level; a third apparently needed 150 mg. for "saturation." Thus it would appear that the intake of ascorbic acid that can be trusted to maintain "saturation" may be considerably above the 75 mg. provided in the recommendation of the National Research Council's Committee. But since, evidently, the individual differences of requirement in this sense may be quite large, it would be well if large numbers of individuals could be studied, on this same general plan, on intakes of, say, 150, 125, 100, 80, 60, and 40 mg. per day.

In Storvick & Hauck's main series, the outstanding data for the two subjects most often tested were as follows:

TABLE II

SATURATION TEST FOR ASCORBIC ACID

Intake level and subject	Urinary ascorbic acid per 24 hrs. mean, *mg.*	Fasting plasma ascorbic acid mean, *mg.* percentage	Response to test dose	
			Ascorbic acid in urine *mg.*	Ascorbic acid in plasma *mg.* percentage
200 mg.				
C.S.	154	1.58	261	1.53
H.H.	163	1.07	267	1.05
125 mg.				
C.S.	82	1.74	207	1.95
H.H.	87	1.05	214	1.27
100 mg.				
C.S.	59	1.32	250	1.71
H.H.	65	0.99	220	1.14
90 mg.				
C.S.	49	1.40	208	1.43
H.H.	58	0.84	228	1.02
55–50 mgs.				
(55) C.S.	29	1.24	92	1.45
(50) H.H.	26	0.85	121	1.03

Noteworthy here is the striking and consistent individual difference in plasma ascorbic acid throughout, notwithstanding close similarity of urinary outputs at all the higher levels of intake; also, that when intake was insufficient to support saturation, the higher plasma level was associated with a lower urinary-output response to the test dose of 400 mg. (given in two portions a few hours apart).

Levcowich & Batchelder (52) studied the urinary excretion and certain other aspects of ascorbic acid metabolism in forty-five young college women. Thirty-two of them, observed in parallel ways in two successive years, showed an average urinary output per day of 17 mg in the first year and 24 mg. in the second. As computed from their dietary records for seven consecutive days preceding and including the day of the 24-hour urinary collection, the coefficient of correlation of the intake and output of ascorbic acid was 0.45 in the first year and 0.48 in the second. Thus the increased urinary output shows a consistent relation to increased intake, even though the computation of intakes by use of data on raw-food values doubtless gives indications higher than the absolute intakes. Quantitative relations of intake and output were then studied more intensively in eight women, and still more fully in two of them, by controlled experiments on a basal diet of low and constant ascorbic acid value supplemented with different amounts of the pure vitamin. The average output of these experimental subjects when receiving 50 mg. per day in a post-saturation period was higher than the average found in either year's survey of the thirty-two-case population sample. Thus the data illustrate objectively the good reasons for further investigations of the conservation of the vitamin values of foods in storage and cooking; such investigations are now in progress. Levcowich & Batchelder also give full data for urinary outputs of two subjects on stepwise varied intakes which again illustrate individual differences in quantitative responsiveness, though the difference was apparently not so large here as between the two subjects intensively studied by Storvick & Hauck as noted above.

Fincke & Landquist (53) investigated further the quantitative relations between levels of intake of ascorbic acid and the resulting concentrations of the vitamin in the blood plasma of healthy young men and women. In their extended experiments with these subjects, attention was largely focused upon the intakes necessary (*a*) to support a plasma concentration level of 0.8 mg. per 100 ml., as generally presumed adequate though perhaps not optimal, and (*b*) to support a

bodily condition of "vitamin C saturation." In this investigation it was found that the daily intake needed to maintain a blood level of 0.8 mg. of ascorbic acid per 100 ml. of plasma was 38 to 49 mg., 49 mg., and 61 mg., respectively, for the three young women studied; and 69 and 89 mg. per day for the two men. Considered in relation to body weight, these requirements for the five subjects are all within the range of 0.8 to 1.2 mg., and they average 1.0 ± 0.14 mg., per kg. of body weight. In three other female subjects it was further found that the daily intakes required to maintain saturation levels were, respectively, 111, 111, and 131 mg. per person, or 1.7, 1.8, and 2.0 mg. of ascorbic acid per kg. of body weight. In general it was found, in this work of Fincke & Landquist, that in graded series of experiments with stepwise variations of intake, the blood plasma level paralleled the intake more closely than did the urinary excretion response to the usual test dose of 400 mg. imposed for a single day, at the end of an experimental period on a given level of intake. Interpretation also gains in clarity when the plan of experimentation permits stepwise variations of intake level without the intervention of the large test dose.

Insofar as "maintenance of saturation" is considered to be the desired condition, interest must attach to the questions, how quickly and for how long a given intake results in an approximately maximal blood level. This has been studied by Todhunter, Robbins & McIntosh (54) whose results hardly admit of brief abstract and should, therefore, be read in the original.

Prevalence of Dietary Inadequacy and Malnutrition

The prevalence of malnutrition and the problem of assessing the nutritional status have been reviewed elsewhere (55, 56, 57) within the year. Here we mention only the dietary survey by the Canadian Council on Nutrition, the Toronto demonstration of the outstandingly significant results of improving prenatal diets, and the cooperative study on "Medical Evaluation of Nutritional Status" conducted in New York City by the Cornell University Medical College, the Milbank Memorial Fund, the New York City Department of Health, and the United States Public Health Service.

Survey by the Canadian Council on Nutrition.—As part of the investigation of the Canadian Council on Nutrition into the status of nutrition in Canada (58), dietary surveys have been made on low-

income (less than $1500 annually) families in Halifax (59), Quebec (60), and Edmonton (61), and on a moderate-income ($1500–$2400) group in Toronto (62). The general plan of the earlier (63) survey of low-income families in Toronto by a local committee was followed. In each city 75 to 100 families participated and under the supervision of trained workers the exact quantity of each kind of food consumed by each individual in the course of one week was measured and recorded. The adequacy of the intakes of calories, protein, calcium, iron, vitamin A, thiamin, and ascorbic acid was judged by comparison with the "Canadian Dietary Standard" (58, 62). Space does not permit reproduction here of the details of the "daily requirements" for calories and for protein so specified. Of calcium, the standard is 0.6 gm. daily for men and for women (with increase to 1.5 gm. during pregnancy and lactation); 1.0 gm. per day for children less than 11 years old; and 1.25 gm. for older children. The iron allowance is 10 mg. for men, 15 mg. for women, 5 mg. for infants, 10 mg. for ages 1 to 5, and 15 mg. for older children. Tentatively, as a basis for comparison of the results of the dietary survey, the adult requirement is set at 6000 I.U. vitamin A, 500 I.U. (1.5 mg.) thiamin, and 1200 I.U. (60 mg.) ascorbic acid. Correspondingly, for children: vitamin A, 100 I.U. per kg. of body weight; thiamin for infants, 100 I.U. (0.3 mg.); thiamin for children 2 to 18 years, 500 I.U. (1.5 mg.); ascorbic acid, 800 I.U. (40 mg.).

All five of these studies demonstrate how fallacious may be the assumption—implicit in dietary surveys of the more usual type, where only the total family intake is measured—that the family's food is apportioned among the various members according to the nutritive need of each. Considering the diet as a whole, it appeared that the men were best fed, followed by the younger children, then by the older children; the women received the poorest diets of all. The exact relations varied, however, for the different nutrients. Thus, among the 76 Quebec families observed, deficiency of vitamin A was the dietary fault most prevalent and most serious in degree in the adults; whereas, among the young children in the same families, vitamin A was the most adequately supplied of the seven factors under consideration. Merely to have stated that the average vitamin A intake of the family was two thirds of the average need would have given an incomplete and somewhat misleading picture of the family status with respect to this factor. Calcium deficiency, on the other hand, was much more common among children than among adults in all the low-income

groups. Of the same Quebec families, the frequency of seriously inadequate (below 70 per cent of the respective standard) calcium intakes was almost twice as great for women as for men, three and one half times as great for children under 11, and more than five times as great for teen-age children as for their fathers.

In all four cities, children of families with low incomes had very low calcium intakes: seriously inadequate, for example, in 85 per cent of the boys and 92 per cent of the girls of teen-age in Quebec. The extent to which cheese was used in the low-cost dietaries seemed to be a large factor in determining the adequacy of their calcium content, for the amount of milk drunk as such was low: in Halifax, a little more than two fifths of a pint per caput daily; in Quebec, about one third of a pint daily for adults and older children, two thirds of a pint for young children. Of particular interest are the findings in Toronto, where (as noted above) observations were made at two different levels of family income. There the average daily consumption of milk in the families below $1500 was 0.26 pint by the men, 0.35 pint by the women, 0.42 pint by children under 11, and 0.37 pint by older children. In the families with incomes from $1500 to $2400, the consumption of milk by the men and children was fully twice as high as in the lower-income group. Correspondingly, the average family intake of calcium was 47 per cent greater, and the incidence of seriously inadequate intakes was reduced to a comparatively unimportant level in all groups except the adolescent girls, of whom 44 per cent received less than 70 per cent of the desirable allowance.

Of iron, the men almost invariably had intakes up to the standard, while the situation appeared distinctly less satisfactory for the women and children. In Toronto, though the higher-income families received 37 per cent more iron than their low-income neighbors, still 44 per cent of the women, 24 per cent of the adolescent girls, and 33 per cent of the younger children had intakes less than 70 per cent of "standard" so that on this basis iron lack was a major shortcoming of the dietaries of the medium-income group. Recent evidence, however, makes the iron content of the food appear a less critical factor in the occurrence of nutritional anemia.

As already indicated, shortage of vitamin A was the most extreme dietary lack noted among the adults in Quebec (where four fifths received less than 70 per cent of "standard") and was also a principal failing in Halifax (half the adult diets supplying less than 70 per cent of "standard"). The situation with respect to this factor was, how-

ever, somewhat better in Edmonton, and in Toronto "quite satisfactory."

In all sections and in every age-and-sex group the intake of thiamin was highly unsatisfactory when judged by the Canadian Standard. In most groups the thiamin intake fell below 70 per cent of the "standard" for more persons than did the intake of any other nutrient studied; in the remaining groups it ranked second in importance as a nutritional problem. Even in the moderate-income families in Toronto, three eighths of the men and half to three fourths of the women and children had "seriously inadequate" intakes of this factor; similar or more unfortunate situations were reported in other sections.

Compared with the standard recommendations, the intakes of ascorbic acid were generally more satisfactory than those of thiamin, but, except perhaps in the teen-age groups, inadequate intakes were quite common. In the higher-income families of Toronto, about a fourth of the younger children, about a third of the men and older boys, and nearly half of the women got less than 70 per cent of their "standard" intake. This was true of less than a twelfth of the teen-age girls, who "in an endeavor to keep slim, show a preference for fruits." The intake of ascorbic acid seemed to be higher in Quebec than elsewhere.

Study of effects of improved diet during pregnancy.—In Toronto, Ebbs, Tisdall, and co-workers (64, 65, 66) have investigated the effect of improved diet during pregnancy on the course of pregnancy, labor, and convalescence of the mother and on the general well-being of the baby during the first six months of life. The food intake for one week was recorded by each of 380 women without major disease and who had not reached the end of their sixth month of pregnancy. To 170 of them, whose diets were moderately good and whose incomes (though low) permitted them to improve their diets, were given advice on still better selection of food. The remainder, who reported much poorer diets in this initial test, were divided into two groups, of which one (120 women) continued on their usual "poor" diet without advice, while to the other (90 women) were given both supplementary foods and assistance in improving their choice of foods from the family table. The supplementary foods included milk, cheese, eggs, oranges, canned tomatoes, wheat germ with added iron salts, and viosterol. They provided daily an average of 45 gm. protein, 46 gm. fat, 60 gm. carbohydrate, 840 Calories, 1.45 gm. calcium, 15 mg.

iron, 50 to 80 mg. ascorbic acid, 350 to 400 I.U. thiamin, and 2000 I.U. vitamin D. Four to six weeks before confinement a second week's dietary study indicated that, as a daily average, the women in the "poor-," "supplemented-," and "good-" diet groups received, respectively: of protein, 62, 94, and 92 gm.; of Calories, 1837, 2424, and 2521; of calcium, 0.736, 1.61, and 1.30 gm.; and of iron, 11.6, 24.3, and 18.3 mg. The women were under observation, in their respective dietary groups, for about four and one half months before confinement. Each woman was rated as to condition and progress during pregnancy, during labor, and during convalescence by an obstetrician who was unaware of the diet group to which she belonged. That the general picture was strikingly improved by better diet is indicated by the obstetrician's report for those on a poor diet throughout that 34 per cent had a "poor" or "bad" pregnancy, delivery, and recovery; while for the group given supplementary foods, the fraction so classified was only 6 per cent; and for those with the initially better diets and nutritional advice, 15 per cent. The improvement attributable to nutrition was strikingly apparent also in such strictly objective findings as that the duration of labor averaged five hours less in the "supplemented" than in the "poor" diet group; that the incidence of anemia was only three fifths and that of toxemia and of infections following delivery, less than one half in the former as in the latter; threatened miscarriage and prematurity were reduced in even greater degree; and there were no miscarriages or stillbirths in the "supplemented" group, as compared with incidences of 6 per cent and 3.4 per cent, respectively, in the "poor" diet group. The women who had had the better diets during pregnancy were subsequently more successful in lactation. The follow-up from birth to six months of the first 250 babies born in this study showed that the incidence of both major and minor diseases was strikingly greater among those whose mothers had had a poor diet before their birth.

Milbank Fund studies in New York City.—Further reports have appeared of the studies on "Medical Evaluation of Nutritional Status" of which preliminary mention was made in the review two years ago by Stiebeling & Leverton (67). Kruse, as already mentioned, has developed and commended the biomicroscopic detection of incipient xerosis conjunctivae as an early indication of vitamin A subnutrition (39). The same examination will also reveal, if present, the keratitis (68, 69) which marks riboflavin deficiency. In both cases, the experienced observer can make also some estimate of the degree of severity

of the deficiency condition. These tests for vitamin A- and riboflavin-lack have now been applied, along with determinations of hemoglobin (discussed above) and plasma ascorbic acid, to several groups in New York City (10). Among some 500 high school students from low-income families, 7.7 per cent showed xerosis conjunctivae which had progressed to the stage of macroscopically visible Bitot spots and 86.6 per cent showed some discernible signs of xerosis. Correspondingly (70), the dietaries reported for a much larger group from the same school were estimated in 65 per cent of the cases to provide less than the "recommended" 5000 to 6000 units of vitamin A daily. Evidence of riboflavin lack was detected in 75.8 per cent of the same group of 500 (10), with about the same proportion (70 per cent) of the larger group (70) reporting diets which provided less than the National Research Council's recommendations for riboflavin. Low-income high school students reported diets which fell below recommendations for ascorbic acid in 58 per cent of the 2000 cases. The fraction of plasma ascorbic acid values below 0.6 mg. per 100 ml. was 47 per cent for this large group (71) and 50 per cent in the smaller group whose vitamin A and riboflavin status has just been reviewed.

Judged by the same criteria of nutritional status, an adult low-income group (W.P.A. employees) showed vitamin A insufficiency in almost 100 per cent of the cases, with "spots" in nearly half; 65 per cent of the men and 38 per cent of the women had blood plasma levels below 0.6 mg. ascorbic acid per 100 ml.; keratitis was only about half as common among the adults as in the high school group.

In contrast with this evidence of widespread subnutrition among the low-income groups, a high-income group of 350 high school students showed blood ascorbic acid levels below 0.6 mg. per 100 ml. in less than 6 per cent and over 1.0 mg. per 100 ml. in nearly three fourths; keratitis was present in only 2.3 per cent of the cases. These studies did not include biomicroscopic examination for A-avitaminosis. A dietary survey (30) presented likewise a much more satisfactory nutritional picture in this than in the lower-income group, with, however, intakes below the National Research Council's recommendations for vitamin A in 44 per cent, and for ascorbic acid and for calcium in about one fifth of the cases.

The large-scale survey of the diets of low-income high school pupils already briefly mentioned (70) was based on interviews both of the mothers and of the children themselves. The questioning covered in detail the food intake for one or two days and included

(among much else) further information as to the average frequency with which certain types of food (e.g., milk, meats, eggs, citrus fruits) were eaten. Though it was realized that reliance upon descriptions and impressions of the size of servings must in the case of the individual dietary yield less accurate figures than actual weighing and measuring of the foods eaten, it was felt that the much larger number of cases which could be covered by this method would present a more representative over-all picture of the dietary habits of this group of children than intensive study of only a few. For each of over 2000 boys and girls the intake of specific nutrients was estimated and compared with the National Research Council's recommendation (see Table I) for that age and sex; the calculated caloric intake was referred to the individual need as computed from body measurements and from the activities reported for the days under consideration. It was obvious that a very large proportion of the children in this low-income group have diets less than desirable in many nutritional regards. The situation appears to be most serious with respect to vitamin A, and only slightly less so for ascorbic acid, calcium, riboflavin, and (among the girls) for iron. As already noted, objective bodily evidence of nutritional deficiency of the three vitamins was found in a large fraction of the group examined. A correspondingly high incidence of anemia among girls, such as the figures for "seriously deficient" iron intakes might lead one to expect, was not encountered (see above); this suggests that the National Research Council's recommendations include a more liberal "margin of safety" for iron than for vitamin A, riboflavin, and ascorbic acid, at any rate for girls 13 to 19.

"A Concept of the Deficiency States"

Under this title, Kruse (72) unifies or integrates the main results of his studies of the effects of shortages of different vitamins and reconciles what some have regarded as discrepancies in the estimates of frequency of poor diets on the one hand and of medically demonstrated malnutrition on the other.

Kruse holds that, while each avitaminosis shows a specificity of the particular tissue sites in which characteristic lesions appear, the changes in each tissue may be quite different, depending on the velocity at which they have developed, whether they are "acute" or "chronic." Each of these categories may be further subdivided on the basis of the intensity of the pathological process, whether it is

"severe" or "mild." Thus we may meet "severe acute," "severe chronic," "mild acute," and "mild chronic" types of deficiency conditions. According to Kruse's view, the rate at which the tissue process develops determines the maximum rate at which any curative force can act. Thus, for example, a sudden change from an adequate diet to one which is markedly deficient in any essential produces rapidly developing or "acute" tissue changes. Application of sufficient therapeutic doses of the essential brings about a reversal of these changes or a cure, which is correspondingly rapid. Most of the avitaminoses produced experimentally in laboratory animals have been of this acute type, and correspondingly we have come too confidently to expect vitamin therapy to produce responses with dramatic rapidity. Similarly, the cases of human riboflavin deficiency so far recognized in the clinic have almost exclusively been acute in nature. This is partly because the rapidly appearing tissue changes and their related symptoms are more apt to call attention to themselves than the insidious slow chronic alterations but partly also because specific therapy with the lacking essential in the latter type, by the very nature of the case, acts with such disappointing slowness as to have been dismissed by the clinician as unsuccessful. However, Kruse holds in the light of recent surveys and the findings of new diagnostic methods that chronic malnutrition must be much more common than acute, and with suitable means of detecting its effects on the tissues the apparent incidence of malnutrition in the general population increases enormously. Thus the biomicroscopic tests (39) for xerosis conjunctivae, which reveal chronic and mild acute changes which would ordinarily escape notice, indicate (as noted above) a far higher frequency of A-avitaminosis than have surveys applying other criteria. Similarly, more recent biomicroscopic examinations of the gums (73) and tongue (74) of 49 low-income adults for manifestations of avitaminosis-C and aniacinosis, respectively, revealed what Kruse considers evidences of both avitaminoses in every case. Each condition responded independently to specific therapy with the factor involved, but even at high dosage levels long courses of treatment (often more than a year) were required for complete tissue restoration; the slow recovery reflected the gradual rate at which the condition presumably developed. In the past, Kruse thinks, many recognized cases of avitaminosis must have been incompletely treated, intensive therapy having been continued merely until the acute symptoms subsided or until the level of the vitamin in the blood reached normal, while the underlying chronic condition of the affected tissues was only partially reversed.

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ANIMAL PIGMENTS

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This year's reviewer has been faced with a difficult task. Global war has not only delayed the arrival of many scientific journals, but it has also cut off the occupied and surrounded countries of Europe from the rest of the civilized world, so that not until peace is restored can we learn of their contributions to our subject. Whatever constructive scientific work may have been performed in the Axis countries has necessarily not been available to the reviewer.

Animal pigments were last reviewed in the 1942 volume, and interest has continued upon much the same lines as were there presented. A need for detecting small rather than gross changes in blood pigment concentration has arisen in connection with problems such as incipient malnutrition, industrial health hazards, the effect of high altitudes and so on, and this has led to a re-examination of the methods available for the determination of haemoglobin and much criticism of their shortcomings. This review will briefly consider such questions.

Interest has also been reawakened in the red pigment of mammalian muscle, myoglobin, since it has been detected in the urine of air-raid casualties who have suffered severe crushing injury of the limbs. Much experimental work is now being done on this condition and the accompanying renal failure.

Accessible papers published during 1942 until the time of writing have been noticed insofar as possible. Pigments other than the haem derivatives have formed a minor contribution and can only be dealt with briefly.

Haemoglobin

Structure.—In view of the strong evidence that histidine participates in the union of globin with haem, precise analytical data for the haemoglobins of different species are welcome. Vickery (1) has developed a quantitative method based on the excellent properties of 3,4-dichorobenzenesulphonic acid as a precipitant for histidine (2) and reports the following percentages for three haemoglobins: human, 8.09 ± 0.04; horse, 7.66 ± 0.16; sheep, close to 7.38. As-

suming a molecular weight of 66,700 for each, in accordance with their similar iron contents, these figures indicate 35, 33, and 32 histidine residues, respectively, per molecule of haemoglobin. Corresponding figures for arginine, determined as flavianate, were 4.21, 3.59, and 3.89 per cent, or 16, 14, and 15 residues, respectively, per molecule. The proportions of these two basic amino acids vary, therefore, in the three species examined, a finding which speaks against the existence of a similarly constituted basic nucleus for proteins of a single type (3).

The crystals of horse methaemoglobin, which have been closely studied by x-ray crystallographic methods (4), are monoclinic with strong birefringence and dichroism; a variety of oxyhaemoglobin exists which is isomorphous with them. Results are now reported (5) for an orthorhombic variety of horse oxyhaemoglobin obtained by crystallisation from twenty per cent alcohol and showing neither birefringence nor dichroism. These needles do not shrink on drying as does methaemoglobin, but they do acquire positive optical character which is lost again on rewetting. The wet crystals are unstable; however, the length of the needle axis was determined as 64 Å, whilst the axes of the dry crystals were a = 77.6 Å, b = 86.8 Å, c = 58.3 Å; space group, P $2_12_12_1$, with four molecules per unit cell. The vector maps show generally close similarity with those of methaemoglobin. Whereas in the latter the molecules all lie in line, the optical properties of these oxyhaemoglobin crystals, when wet, could be explained by picturing the two pairs of molecules in each unit cell tilted, with regard to one of the axes, at angles of equal magnitude but opposite sense. The reversal of the sign of the birefringence on drying could be brought about by a slight alteration in the tilt.

Oxygen dissociation.—Haemoglobin, with a molecular weight of approximately 67,000 in neutral aqueous solution, contains four haem groups each capable of additive combination with one molecule of oxygen. It is well known, however, that in the classical equation

$$\frac{[\mathrm{Hb}]_n \times [\mathrm{O_2}]^n}{[\mathrm{HbO_2}]_n} = K,$$

which represents this relationship, the value of n is not 4 but 2.43. Coryell (6) suggests that this discrepancy may be occasioned by a step-wise, rather than uniform, addition of oxygen. The value, n = 1, holds in the corresponding equation for myoglobin, in which there is only one haem residue per molecule (mol. wt. approx. 17,000).

Taylor & Hastings (7) have investigated the oxygen dissociation curves of haemoglobin dissolved in 4 *M* urea solution, since under these circumstances dissociation into half molecules is known to occur. The value of n was found to be 2.9, indicating a lack of strict correlation with the molecular weight.

The oxygen dissociation curve of haemoglobin has also been investigated from another angle which has an important physiological bearing. The toxic effect of methaemoglobinaemia or carboxyhaemoglobinaemia is recognised to be greater than that of an equivalent degree of anaemia; as Haldane (8) expressed it,

> Miners may be doing their ordinary work though their haemoglobin percentage is reduced to half or less by ankylostomiasis—whereas a person whose blood is half saturated with carbon monoxide is practically helpless.

The physiological difference in the case of carbon monoxide poisoning was shown (9, 10) to be largely due to the fact that the oxygen dissociation curve of the remaining haemoglobin becomes more hyperbolic, thus losing the advantage in combining power at low oxygen tension which its natural sigmoid form had conferred upon it. The present study (11) shows that the same effect is produced, although in lesser degree, by methaemoglobin. The suggested explanation of this mechanism is that oxidation takes place in steps, one iron atom at a time, and that the presence of one ferric atom may increase the affinity for oxygen of the remaining three ferrous atoms in the molecule. In any case the difficulty which methaemoglobin-containing blood has in unloading its oxygen should warn us to view industrial cyanosis somewhat more seriously than we are inclined to do at present.

Methaemoglobin and haemoglobin system.—That an interaction between the four haem groups does occur when methaemoglobin is progressively reduced to haemoglobin and is responsible for deviation of the oxidation-reduction potentials from the theoretical electrode equation $E_h = E'_m + \frac{RT}{nF} \ln \frac{[\text{methaemoglobin}(Fe^{+++})]}{[\text{haemoglobin}(Fe^{++})]}$ is suggested by Taylor's (12) measurements upon this system. In aqueous solution the value of n[1] for haemoglobin progresses from 1 to 2 as titration proceeds. In the case of metmyoglobin-myoglobin (13) the electrode equation is obeyed with $n = 1$; hence it was of interest to re-examine

[1] n is the number of electrons involved in the electrode reaction and is not to be confused with "n," the numerical constant in the Hill equation.

the haemoglobin system in 4 *M* urea solution in which dissociation to half-molecules occurs.

A constant value of $n = 2$ was obtained (14) which is consistent with the supposition that the pairs of haem groups are reduced "at the same time." Explanation, however, is still needed for the changing values of n obtained in the absence of urea, especially since dog haemoglobin behaves similarly to horse haemoglobin under these two sets of conditions; Wu & Yang (15) have stated that, unlike horse haemoglobin, it is not dissociated in urea solution (osmotic pressure measurements).

The potential of an equimolecular mixture of horse haemoglobin and methaemoglobin in 4 *M* urea is stated (14) to be +0.108 v. at 30° and pH 7, whilst the corresponding value for the horse myoglobin system is +0.046 v.

In the circulating blood, methaemoglobin gradually reverts to functional oxyhaemoglobin. A comprehensive study of the rate of this reaction and of the effect upon it of the administration of various substances is presented by Cox & Wendel (16) who conclude that the transformation is brought about mainly, if not entirely, by enzymes in the circulating erythrocytes.

Fresh blood of man, cat, dog, rabbit, rat, mouse, horse, ox, pig, and *Macacus rhesus* monkey was found to contain no spectroscopically demonstrable methaemoglobin (sensitivity 4 per cent of total pigment). In dogs, intravenous injection of drugs, such as sodium nitrite and *o*- and *p*-aminophenols, causes easily reproducible degrees of methaemoglobinaemia and the rate of disappearance of methaemoglobin is rapid, the same in each case and, moreover, independent of the total pigment concentration. Thus, after 0.5 cc. of 6 per cent solution of sodium nitrite per kg., the mean rate of disappearance was 11.2 per cent of total pigment per hr., standard deviation ± 2.0 per cent. Lowering of body temperature by 10° decreased the rate to only 3.3 per cent. Variations in blood sugar level or the administration of glucose have no effect, neither has fasting nor a long-maintained "chronic" methaemoglobinaemia. Methylene blue accelerates the rate of disappearance, but after organic drugs such as aniline, the effect may be only temporary, due to the slow conversion of these drugs into the active agent. This explanation is borne out by the fact that the duration of methaemoglobinaemia after aniline is more prolonged than after a comparably effective dose of a simple agent.

The capacity of ascorbic acid to reduce methaemoglobin *in vitro*

to the extent of 80 to 90 per cent in six hours (17) is in accordance with the overlapping oxidation-reduction potentials of the haemoglobin and ascorbic acid systems. Methylene blue accelerates the rate of reduction. Nitrite methaemoglobin is only very slowly reduced and appears to differ from the pigment produced by ferricyanide.

The reaction of cyanide with nitrogenous derivatives of ferriprotoporphyrin (18) was discussed in last year's review (19).

When solutions of oxyhaemoglobin are acidified, not all the oxygen is liberated; oxidative change is known to occur in both haem and globin portions of the molecule. Lemberg (20) has shown that active oxygen is first formed, capable of rapidly oxidising ascorbic acid if present. In the absence of added substances, globin acts as the hydrogen donator, resulting in the production of hydrogen peroxide which in turn causes a small destruction of haematin with release of "easily detachable iron." The attachment of the prosthetic group is also altered so that some of the haematin becomes firmly bound (21). There are thus two oxidising systems produced, an evanescent one due to the liberation of active oxygen, and a less rapidly disappearing hydrogen peroxide-haematin one (studied quantitatively by the transformation of added biliverdin into biliviolin). Addition of ascorbic acid before acidification will provide an acceptor for the activated oxygen and hence prevent the establishment of the secondary system and the changes noted above. Suitable precautions should be taken before deproteinizing solutions containing oxyhaemoglobin and any easily oxidisable substance which it is desired to estimate.

Haemoglobin determination. — Of physiological determinations that of the quantity of haemoglobin in the circulating blood is one of the most important. It is surprising, therefore, that now that interest has shifted from gross variations from the normal to the relatively slight changes which may be indicative of a lowered standard of nutrition, effect upon blood donors of periodical bleeding, early intoxication by materials handled in industry and so on, all the methods which have been in use by haematologists should prove to be subject to serious disadvantages and errors. The last decade has seen the widespread adoption into biochemistry of the photoelectric colorimeter in one design or another, but little advantage is gained by introducing this instrument into haemoglobinometry unless the colour reaction measured is in itself sound. The position has been aggravated by wartime difficulties in obtaining the relatively expensive photoelectric apparatus. Haematologists, moreover, have often ex-

hibited a tenacious conservatism as far as the particular method which they favoured was concerned.

A brief review by the writer (22) has set forth some of the advantages and disadvantages of each of the principal methods. Some, such as the gas-combining power techniques, measure only the functional haemoglobin. It may be argued that this is really the figure in which the physiologist and clinician are primarily interested and that the proportion of nonfunctional pigment may be disregarded. In a severely cyanosed patient, such as one suffering from sulphonamide intoxication, however, a totally erroneous impression of the state of the blood would be given by gas-binding measurements alone. Another difficulty is that in every instance one must either calibrate an instrument or method with pure haemoglobin, or adopt some figure quoted in the literature for the particular function of pure haemoglobin upon which the method is based. The chemical determination of iron in the washed red cell would appear to be one of the most dependable methods, but is somewhat too tedious for adoption as a routine clinical method.

The errors affecting the acid (Sahli) and alkaline haematin (Wu) methods have been again investigated by Ponder (23). Results often showed poor agreement with haemoglobin as determined by Wong's iron method. The chief sources of error are the long and variable times required for colour development and the effect of substances from both cells and plasma on the final intensity.

Clegg & King (24) propose heating the solution of blood in alkali for five minutes in the boiling water bath to accelerate colour development and maintain that the method then gives good results. Their figures show, however, that the margin of safety in the time of heating is not large. They also propose using solutions of crystalline haemin in alkali as comparison standards but find that blood or haemoglobin gives about thirty per cent more colour than the equivalent amount of haemin. This finding, for which they are unable to offer an explanation, accords with unpublished experience of the writer, and it seems to him that it may be due to the formation of compounds with the denatured globin which might well be expected to have spectral absorption quantitatively different from that of pure haemin in alkali.

No such objection can be raised against the pyridine haemochromogen method (25, 26) recently advocated by the writer (22). According to this technique blood is diluted with alkali, and then

one fifth of the volume of pyridine is added. In the presence of the organic base the transformation into pyridine ferriprotoporphyrin is rapidly completed at room temperature and by the addition of a few mg. of sodium hydrosulphite, pyridine haemochromogen (pyridine ferroprotoporphyrin) is produced with its intense absorption based at about 550 mμ. Quantitative determination may follow in a photoelectric colorimeter with a suitable filter or in an instrument such as the Hilger-Nutting absorptiometer, in which a refracting prism allows the selection of light of any desired wave length. In any case the instrument may be calibrated using crystalline haemin similarly converted into pyridine haemochromogen, since the intensity of absorption of this pigment, unlike that of "alkaline haematin," is the same whether derived from blood or pure solution. Crystalline haemin thus becomes an absolute standard. Should photoelectric apparatus not be available, a simple colorimeter may be used and a comparison solution made from haemin or a subsidiary artificial standard either of glass or prepared from phenol red (Rimington, unpublished work) may be employed. Results, using this method, have shown much promise.

Several designs of simplified visual and photoelectric haemoglobinometers have been prepared, some (27) utilising alkaline haematin, others (28, 29) the unchanged haemoglobin. In all instances the fundamental difficulty is that of calibration.

Haemoglobin regeneration.—The main results of the fundamentally important work emanating during the past years from Whipple's laboratory upon haemoglobin production and regeneration in dogs have been summarised in two general articles (30, 31). The problem is intimately bound up with that of nutrition and protein metabolism in general, but it is interesting to note that haemoglobin production in the animal rendered artificially anaemic will take precedence over plasma protein production, even should the dietary protein supply be less suited to the former (30).

That a reserve of haemoglobin-building material is held in the liver seems clear from these studies and the variations of this reserve in the human in connection with conditions such as pernicious, aplastic, and secondary anaemias, haemolytic icterus, polycythaemia, leukaemias, pregnancy, eclampsia, and hypoproteinaemia are further explored in a recent paper (32). Exhaustion of the protein stores lowers the barrier to infection and renders the liver liable to injury by many toxic substances. One cannot help realising the significance

of this fact when contemplating the ever increasing severity of malnutrition in the unfortunate Axis-occupied countries of Europe. Relief of nutritional hypoproteinaemia should be one of the primary aims in plans for medical assistance, and these studies offer significant dietary guidance.

Haemoglobinuria.—Haemoglobinuria has been reviewed by Yuile (33) who marshals evidence to show that glomerular injury is not the fundamental mechanism of haemoglobin excretion. An explanation is favoured depending upon the observed variation in size of the pores in the glomerulus of the frog (34), and it is pointed out that the low rate of haemoglobin excretion relative to its concentration in mammalian plasma indicates that under maximal conditions only about three per cent of the glomerular pores are large enough to allow the haemoglobin molecule to pass through.

Myoglobin

The appearance of a haemoglobin-like pigment in the urine of patients who have suffered crushing injury of the limbs in air-raid accidents, and in whom renal injury and shock frequently develop, has led to suggestions that the pigment might be myoglobin derived from the injured muscles. The identity has been proved spectroscopically and the deposits found in the kidney tubules shown to be a haem derivative convertible into pyridine haemochromogen (35). What part, if any, myoglobin may play in the development of the renal lesion is as yet uncertain, but the reader may be directed to certain further papers (36, 37) dealing with this matter. It is of interest to note, in connection with the discussion of haemoglobinuria, that myoglobin with a molecular weight one-quarter that of haemoglobin is rapidly excreted by the kidney, so that the plasma may appear of normal or nearly normal colour, even when the urine is a deep reddish brown. The dielectric properties of horse myoglobin have been studied by Marcy & Wyman (38).

A somewhat remarkable claim has been made (39) that myoglobin can be reversibly transformed into cytochrome, but Fischer & Gibian (40) point out that the alteration in spectrum, upon which the claim is based, might possibly be brought about by an action upon the vinyl groups of the pyridine-hydrazine hydrate reagent used.

The course of pigment metabolism following the transfusion of freshly drawn and stored bloods, respectively, into normal human beings has been followed by Vaughan (41), who reports a rise in serum

bilirubin up to 5 mg. per cent after three weeks old blood, but no significant difference following fresh blood. In either case the increase in faecal urobilinogen was slight and it is suggested that any pigment liberated from the old blood is largely retained in the body for subsequent use.

In a further study of some invertebrate erythrocruorins, Salomon (42) has obtained haemin, identical with that of vertebrates, from *Glycera dibranchiata* blood.

Porphyrins

Porphyrins and diet.—Considerable work continues to appear relating to the porphyrins in health and disease. The presence of a porphyrin, considered to be protoporphyrin, in the encrustation adhering to the nose and whiskers of rats deprived of the "filtrate factor" was first reported in 1940 (43) and again a year later (44) as a result of feeding diets deficient in pantothenic acid. That a similar porphyrin-rich encrustation appeared when pantothenic acid requirements were met, but when water was withheld, was observed by Figge & Atkinson (45) and by Smith (46). Figge & Salomon (47) have recently been able to demonstrate by ablation experiments that the porphyrin originates in the Harderian gland, long known to be rich in these pigments (48, 49, 50), the secretion of which bathes the conjunctiva and then normally passes via the naso-lacrimal duct into the nasal cavity. Ultimately it passes into the alimentary canal, where it may account for the relatively large amount of protoporphyrin IX (51) excreted by the rat in its faeces. Should water be withheld, the excretion becomes gluey and remains around the eyes and nose, from which sites it may be smeared over the face and fur by the action of the paws in an effort at cleaning.

In a continuation of studies upon the porphyrin excretion of cattle in health and disease (52, 53, 54), Roets (55) reports that after feeding blood meal there was a rise in both coproporphyrin and chloroform-soluble porphyrin of the faeces, together with a smaller increase in urinary coproporphyrin. This may have arisen from the conversion of absorbed protoporphyrin into coproporphyrin in the liver. No chloroform-soluble porphyrin was present in the urine.

Quantitative methods.—Methods for the determination of porphyrins have received some attention. It has long been realised that the extraction of coproporphyrins from urine by ether in quantities

sufficient for subsequent spectrophotometric determination is too time-consuming and too extravagant in the use of solvent for routine clinical procedure. To overcome this, fluorimetric methods have been proposed (56), but have met with little enthusiasm on account of the high cost of the apparatus. More recently, cheap and convenient ultraviolet sources have become available and interest in the problem has been renewed (57, 58, 59). An inexpensive fluorescence comparator has been designed by Rimington (60) and the optimal conditions for porphyrin determination defined. In particular it is shown that in solutions more acid than about pH 1, the intensity of fluorescence decreases as the acidity increases. This may lead to serious errors where 5 per cent hydrochloric acid is used to extract the porphyrin; a concentration of 0.25 per cent is preferable.

Porphyrin excretion and hepatic insufficiency.—A considerable amount of evidence has supported the view that damage to the parenchymatous cells of the liver is associated with increased urinary excretion of porphyrin, but demonstration has been lacking that the effects are quantitatively comparable. Two papers by Nesbitt & Snell, therefore, are very welcome. In the first (61), seventeen cases are considered, the hepatic injury being assessed by determinations of serum bilirubin, bromosulphalein retention, hippuric acid excretion, and, when available, post-mortem findings; the values for plasma proteins, prothrombin clotting time, and the Van den Bergh reaction were also taken into consideration. In many of these cases porphyrin excretion varied largely from day to day, but sufficient determinations were made to provide a reliable mean. The results are reproduced in Table I.

Four out of five cases with mild liver damage had a urinary porphyrin excretion of between 100 and 200 μg. per day; the fifth was normal, as also was the faecal porphyrin in four cases. Moderately severe damage was assessed in five cases and in four of these the urinary figure lay between 200 and 300 μg. per day, in three cases the faecal coproporphyrin being low. Lastly, of seven cases where the hepatic injury was severe, five had a urinary porphyrin excretion between 300 and 600 μg. per day (the other two lay between 200 and 275 μg. per day) and in six cases the faecal coproporhyrin was distinctly low (100 to 260 μg. per day). It thus appears that the degree of porphyrinuria, and especially the urinary/faecal porphyrin ratio, offer a fair index to the actual degree of parenchymatous damage to the liver. The data are not, however, of prognostic value.

TABLE I

CLINICAL AND LABORATORY DATA IN HEPATIC DISEASE

Case No.	Diagnosis	Liver Damage*	Days Observed	Excretion of Coproporphyrin, μg			Urinary: Faecal Ratio
				In Urine		In Faeces, Mean	
				Range	Mean		
1.	Obstructive biliary cirrhosis; stricture of common duct	++	6	132–500	350	...	...
2.	Obstructive biliary cirrhosis; stone in common duct	+	21	50–178	105	...	...
3.	Hepatitis (syphilitic); biliary cirrhosis; stone in common duct	+	9	76–280	155	312	0.49
4.	Epidemic hepatitis	++	17	58–248	220	...	...
5.	Hepatic metastasis from carcinoma of rectum	+++	8	190–520	275	246	1.14
6.	Fatty liver (gastrocolic fistula)	+	51	60–240	155	350	0.44
7.	Subacute atrophy of liver.	++	49	115–600	275	203	1.40
8.	Atrophic cirrhosis of liver (unknown causation).	++	6	135–445	300	255	1.20
9.	Atrophic cirrhosis of liver (unknown causation).	+	4	18–39	33	395	0.084
10.	Atrophic cirrhosis of liver (unknown causation).	++	17	100–630	270	208	1.3
11.	Latent or compensated alcoholic cirrhosis	+	6	106–242	175	310	0.56
12.	Alcoholic cirrhosis	+++	10	53–417	350	245	1.4
13.	Alcoholic cirrhosis	+++	5	400–782	570	172	3.3
14.	Alcoholic cirrhosis	+++	93	100–600	300	248	1.2
15.	Alcoholic cirrhosis	+++	9	290–844	510	...	...
16.	Alcoholic cirrhosis	+++	48	414–838	595	165	3.6
17.	Alcoholic cirrhosis	+++	92	21–560	200	205	0.99

* + = Slight hepatic damage.
++ = Moderate hepatic damage.
+++ = Severe hepatic damage.

Mechanical obstruction to the flow of bile, as by stone in the common bile duct, may or may not be associated with damage to the liver parenchyma and herein may lie the explanation of the conflicting evidence concerning urinary porphyrin excretion in this condition (62). Nesbitt & Snell (63) have contributed a study which materially helps to clarify the position. Six cases with varying degrees of obstructive jaundice and of parenchymatous liver damage were in-

vestigated by the method outlined above. Before operation the ratio of urinary to faecal porphyrin was raised, and after surgical procedure to restore the bile flow, it remained elevated or returned to normal according to the degree of hepatic parenchymatous damage.

Porphyrinuria and industrial toxicology.—Within recent years several industrial chemicals have been added to the list of poisons that cause increased urinary porphyrin excretion (64, 65, 66). Trinitrotoluene (T.N.T.) also belongs to this group (67) and it appears possible that the property they possess in common is an injurious action upon the liver parenchyma. Kennedy & Ingham (68) proposed a rapid qualitative method for detecting porphyrin in the urine of T.N.T. workers, but its reliability has been disputed (69, 70).

Lead poisoning has long been known to cause a very large increase in porphyrin excretion, an increase altogether larger than would be anticipated from the degree of hepatic involvement or anaemia. Rimington (71) suggested that lead, in some way, prevents the incorporation of iron into the protoporphyrin molecule during haemoglobin synthesis. Waldenström (72), on the other hand, prefers to regard the massive excretion of porphyrin as a result of disturbed cytochrome or other haematinic respiratory pigment metabolism.

Björkman (73), in experimental plumbism, has found no relation between the porphyrin excretion and either liver function or haemoglobin metabolism. After a critical review of existing theories and experimental work, he comes to the conclusion that Waldenström's suggestion is that to be preferred. Kark & Meiklejohn (74) similarly find no disturbance in haemoglobin catabolism and no evidence of increased haemolysis in two human cases of lead poisoning, their view being that the porphyrinuria is probably connected with disturbed haemoglobin synthesis. A third study of industrial lead poisoning by Kench, Gillam & Lane (75) presents some valuable new data including determinations of the non-haemoglobin iron, the concentration of which appears to be markedly raised. They consider this phenomenon and the occurrence of increased blood protoporphyrin to be incidental to restricted cellular activity in general. Diminution in the quantity of haemoglobin is regarded as a consequence of restricted formation of this pigment, not of nonutilisation of protoporphyrin, and they do not accept Rimington's hypothesis of the prevention of incorporation of iron.

It will be realised that whilst all the recent communications add materially to our knowledge of lead poisoning and of the accompany-

ing porphyrinuria, which is such a characteristic factor, the problem of the origin of this porphyrin and the nature of the essential lesion still awaits a satisfactory explanation.

Porphyria.—Little progress has been made towards an understanding of the chemical pathology of acute idiopathic porphyria since Waldenström's (76) finding that both uroporphyrin III and the "red pigment," porphobilin, may be derived from a colourless dipyrrimethene precursor, "porphobilinogen." This observation helps to explain the absence, *post mortem,* of porphyrin accumulations from the organs of the body. The condition itself is now becoming more generally recognised as a clinical entity (77) and a further report has been made during the year of two cases from the Mayo Clinic (78). It is noticeable that no constant ratio is observed between quantities of uroporphyrin and coproporphyrin in the urine.

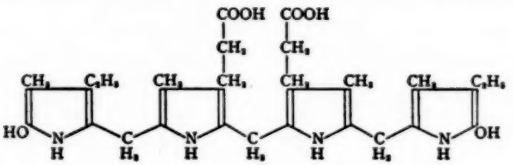
Bile Pigments

Stercobilin and urobilin.—A series of four papers by Watson and collaborators (79, 80, 81, 82) helps to clear up the confusion surrounding "stercobilin" and "urobilin." It will be remembered that reduction of bilirubin by sodium amalgam gives rise to the well characterised mesobilirubinogen. In air this reoxidises to an optically inactive pigment termed "urobilin IX*a*," whilst stercobilin from faeces or "urobilin" of urine (identical with stercobilin) (83, 84) is strongly laevorotary. Amalgam reduction of stercobilin yields stercobilinogen and not mesobilirubinogen.

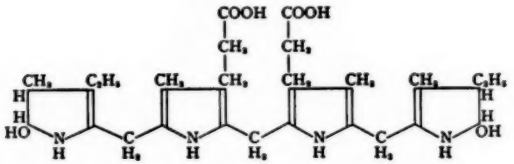
A possible explanation of these differences would be the existence of more than one bilirubin in bile. Whole human bile has, therefore, been reduced (after removing any native urobilinogen) and the product formed on aerial reoxidation identified as the optically inactive urobilin IX*a*. Stercobilin must consequently owe its formation to some chemical activity peculiar to the lower intestinal tract; hence the proposal (85) to exclude this name from the literature cannot be upheld. Incidentally, this experiment affords support for the view that opening of the porphyrin ring occurs only at the α-methene bridge. The finding of Lemberg *et al.* (85) is confirmed that the absorption band of stercobilin is situated about 25 Å nearer the blue end of the spectrum than that of urobilin IX*a*.

A slight spectral difference between the urobilin formed in bacterially infected bile and that of urine was observed as long ago as 1881 by MacMunn (86). The former has been isolated in crystalline

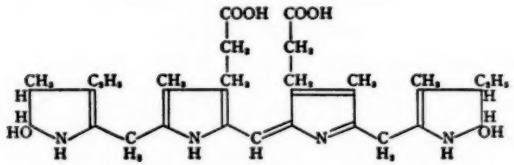
form (80) and found to be spectrally similar to urobilin IX*a* but dextrorotary. It has been tentatively termed *d*-urobilin. That it can arise from mesobilirubinogen has been demonstrated (81) by adding



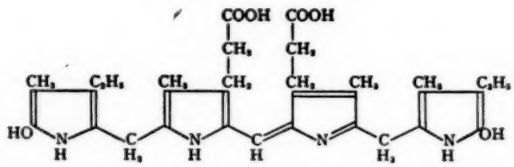
Mesobilirubinogen



Urobilinogen (stercobilinogen)



Urobilin (stercobilin)



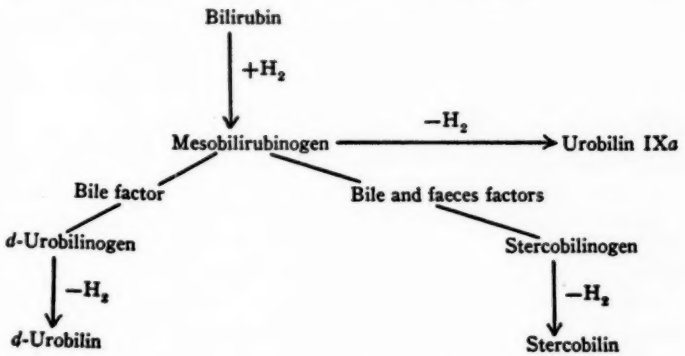
Urobilin—IX*a*

FIG. 1 [after Lemberg *et al.* (85)]

the latter substance to infected bile or to bile plus acholic faeces. That a faecal or alimentary canal factor is necessary for the production of stercobilin from mesobilirubinogen is similarly demonstrated by its

isolation after incubating normal faeces or acholic faeces plus bile with mesobilirubinogen. With acholic faeces alone, only urobilin IX*a* results. Urine is inactive.

To sum up these investigations, mesobilirubinogen appears to be the primary chromogen or "urobilinogen" formed when bilirubin is reduced by bacteria. Reoxidation would lead to urobilin IX*a*, but with the cooperation of a bile factor the secondary product, *d*-urobilinogen, is formed; alternatively, the cooperation of a bile and a faeces factor produces the secondary product, stercobilinogen, thus:



Intracorpuscular formation of bile pigment.—Although the formation of bile pigment within the red cell has appeared probable, rigid proof of this activity has been lacking. Lemberg & Legge (87) have now detected increased quantities of biliverdin and choleglobin in the erythrocytes of rabbits under the influence of phenylhydrazine, an effect which was not due to haemolytic action.

Normal human blood contains 0.4 to 2.0 μg. biliverdin per cc. and so far abnormal values have only been found in a case of erythroblastosis foetalis without clinical icterus where 23 μg. biliverdin per cc. were recorded.

Tests for bilirubin.—Since Van den Bergh's introduction of the direct and indirect colour tests for bilirubin, much work has been performed in efforts to differentiate between different types of icterus by such simple tests (88). Some recent authors (89, 90) have further examined the Van den Bergh reaction. Maher (91) proposes a test using *p*-toluenesulphonic acid and sodium nitrite which, it is stated, gives marked differences in reaction with various types of bile.

Catalase.—Improvements in methods for isolating crystalline catalase are reported by Dounce (92). The inhibition of catalase action by hydroxylamine and *p*-hydroxylaminobenzenesulphonamide and its reversal by serum, serum albumin, or by haemin have also been studied (93). These substances combine with haem pigments as well as with protein and are, therefore, able to inhibit both haem and non-haem types of enzymes.

Cytochrome.—A new member of this group, "cytochrome b_2," has been prepared from yeast by Bach, Dixon & Keilin (94). It has a haemochromogen type of spectrum ($\alpha = 5570$ Å; $\beta = 5300$ Å) and contains a protohaematin nucleus since it is convertible into pyridine haemochromogen. It thus resembles the *b*, but differs from the *a* and *c* cytochromes. It is slightly autoxidisable in solution, but does not combine with carbon monoxide [compare (95)].

This cytochrome cannot replace the substance linking cytochrome *c* with the succinic acid system, but its identity with the lactic dehydrogenase of yeast seems possible (96).

Improved methods have been described for the preparation of cytochrome *c* (97) and of cytochrome reductase (98), and the determination of cytochrome *c* has also received attention (99).

Dietary deficiency and pigment excretion.—Some preliminary notices of great interest have appeared during the year which deal with disturbances of pigment metabolism and suggest interrelations which may prove to have far-reaching consequences. Pantothenic acid deficiency has already been mentioned (47), but the effect in this instance upon porphyrin elimination would appear to be merely an incidental result of disordered water metabolism.

Pyridoxin (vitamin B_6) requirements are known to have some bearing upon haemoglobin production in the animal body. It has now been observed (100, 101) that the urine of pyridoxin-deficient rats or dogs forms with iron salts a green pigment which appears to be neither a porphyrin nor bile pigment derivative. It behaves as an indicator dye and is decolorised by oxidising or reducing agents. The spectral absorption curve shows a maximum at 6250 Å. Elucidation of the nature of this pigment will be awaited with interest.

Coulson, Ellinger & Platt (102) have observed a dependence of excretion of a fluorescent urinary pigment (identified provisionally as thiochrome) upon the level of intake of nicotinamide, which suggests some interrelationship between the functions of nicotinamide and thiamine, and also a possible method for assaying the former (103).

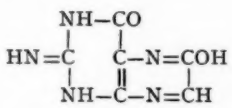
A synergistic effect between tocopherol and vitamin A or the provitamin, carotene, is postulated (104) for the rat.

Vitamin A supplements lower the carotene levels in the blood and milk of cows; the butter vitamin rises in a proportion representing about three per cent of the supplement (105). An interesting investigation by Popper & Chinn (106) dealing with choline deficiency and fatty liver shows *inter alia* that the storage of vitamin A in the liver depends not only upon suitable carotene ingestion but also upon the choline intake. The livers of choline-deficient rats, although rich in fat, were exceedingly low in vitamin A; the renal cortex, however, contained relatively high concentrations.

It has been suggested that an avitaminosis B might account for the neurological symptoms in pernicious anaemia, the achlorhydria leading to decreased absorption. The hypothesis, however, was not supported by measurements of the riboflavin excretion of such patients after test doses of the substance: there was no difference between patients and controls (107).

The riboflavin content of the liver is markedly increased during digestion, presumably as a result of mobilisation from other tissues in normal animals. Thiamin and pantothenic acid deficiencies both prevent this increase and both substances have an augmenting effect if injected (108). An interrelationship between the functions of these factors appears to be indicated.

Pterins.—Since Hopkins' (109) work over fifty years ago on the pigments of the wings of pierid butterflies, much time has been spent, particularly by Wieland's school, upon their investigation, but with little positive result. Since 1940, however, events have taken a dramatic turn and both leucopterin and xanthopterin have been synthesised (110, 111). They are now regarded as simple compounds allied to uric acid, but containing a six-membered ring in place of the iminazole ring of the purines. Xanthopterin, for example, is formulated by Purrmann (111) as:



No less arresting is the evidence suggesting that the pterins may play a vital part in haemoglobin synthesis. Tschesche & Wolf (112, 113) make the remarkable claim that injection of 10 μg. of xanthopterin causes a considerable new formation of red corpuscles in rats made

anaemic by feeding on goats' milk. Jacobson (114) has shown that the argentaffine cells of the normal intestinal epithelium contain a yellow pterin while the location of these cells agrees with that of the substance active against pernicious anaemia. The pigment is absent in cases of this disease. Lastly, Simmons & Norris (115) report that the anaemia of fish fed on a deficient diet is relieved by the injection of either natural or synthetic xanthopterin. Xanthopterin is identical with uropterin isolated by Koschara (116) from normal urine.

It is of considerable interest, therefore, that Hopkins has now returned to his studies of the pterins; he has published a contribution (117) in which the chemistry is described of a coloured oxidation product formed from many pterins in acid, but not in alkaline solution. This purple-red pigment, now termed "rhodopterin," was first described by him nearly fifty years ago under the name lepidoporphyrin. It may prove of great value in elucidating the structures of different members of the group. Of great interest is also the fact that the pterin-like products formed by heating uric acid in sealed tubes are similarly oxidised in acid solution with the production of rhodopterin or of materials of chemically similar nature.

Malaria pigment.—The pigment of malarial parasites has been extracted and examined spectroscopically and also transformed into crystalline haemin (118), thus confirming the observations of Sinton & Ghosh (119) who identified it as haematin.

Other pigments.—Other interesting studies of naturally occurring pigments can only be referred to by title (120, 121, 122, 123).

Since this review was compiled, the writer's notice has been called to an important paper by H. Fischer & C. G. Schröder (124) in which, under the title "Synthesis of Rhodoporphyrin—carboxylic acid anhydride; synthetic rhodins and verdins," they retract H. Fischer's claim (125) to have proved the existence of isomeric haemins in blood as revealed by the production of an isomeric mixture of mesoporphyrins from blood haemin by chemical means (126).

Fischer's claim would have meant that "haemoglobin" contained some pigment molecules derived from aetioporphyrin I in addition to those from aetioporphyrin III and might thus have explained the existence in urine, faeces, etc., of porphyrins of series I and III. The conclusions drawn from the meagre data published in this preliminary report must have been fallacious since Fischer & Schröder have now converted natural mesoporphyrin and synthetic mesoporphyrin IX (aetioporphyrin series III) into their corresponding rhodins and verdins which were found to be identical each to each. No evidence of the presence of mesorhodin II or mesoverdin II was obtained as would have been the case had natural mesoporphyrin contained some mesoporphyrin II (aetioporphyrin series I).

Attention is drawn to the communication, the title of which is hardly such as to reveal its importance relative to the subject in question.

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SYNTHETIC DRUGS

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Although selected portions of the subject matter of this section have been presented previously in reviews of related fields of study, this is the first volume of the *Annual Review of Biochemistry* to include a chapter on synthetic drugs *per se*. Therefore, it appears desirable to limit the number of classes of pharmacological agents to be covered in order to reserve space for the consideration of some of the general factors that may govern the biological activity of a chemical agent. An appreciation of these factors is a necessary prerequisite to the intelligent selection of compounds for synthesis and pharmacological appraisal. Of the many classes of medicinal agents that might be considered and that justly deserve attention, only the antibacterial agents and the local anesthetics have been chosen for consideration. This choice has been dictated, in part, by the limitation of space, and in part, by the limitations of the author. The sequence of events in the development and appraisal of a new synthetic drug necessitates considering agents that are in various stages of the developmental process.[2] However, an attempt has been made to include only those substances that have been subjected to some form of biological evaluation. No attempt has been made to cover the patent literature.

General Considerations

Early in the development of synthetic drugs much emphasis was placed upon the relation that is presumed to exist between chemical constitution and pharmacological action. A number of classes of

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[2] In the development of synthetic drugs a chain of events is involved which usually starts with the synthesis of a compound or series of compounds related chemically either to a naturally occurring therapeutic agent or to a synthetic class of compounds of established pharmacological interest. The new compound, or compounds, is then appraised pharmacologically. This is followed by clinical trial and evaluation. Several years of study are commonly involved before a synthetic chemical can become established as a reliable drug.

chemical compounds tend to support such a view. However, with the continued development of organic chemistry and pharmacology, it became apparent that many compounds not at all related chemically were capable of eliciting the same pharmacological response. Thus, a large number of types of chemical substances are known that are capable of acting as anesthetics, hypnotics, analgesics, etc. Schulemann (1) has pointed out the difficulty of finding "any connection between chemical compounds so differently constituted but having a similar biological action." It is known, however, that many classes of compounds with a given pharmacological action have in common certain physical properties.

Arrhenius (2) made one of the early attempts to interpret the action of drugs through the application of the principles of physical chemistry. Such attempts at correlation suffer from the general criticism that reactions occurring in complex colloidal systems are not comparable to the simple homogenous systems from which physical chemical laws are derived. Despite this criticism, the use of physical chemistry in the problem of correlating the activity of a drug with its structure has yielded excellent results. In the case of a series of compounds wherein the change in structure involves only an increase in length of the carbon chain, gradations in the intensity of action have been observed for compounds in diverse groups of pharmacological agents in which the activity depends upon undissociated molecules. In general, the lower members of the homologous series show little or no activity; with increasing length of the carbon chain the effect increases, passing through a maximum. Further increase in the number of carbons results in a rapid decrease in activity. The increase in activity in an ascending series parallels the decrease in water solubility, the increase in lipid solubility, and, to a certain extent, the increase in surface activity. Such changes in physical properties may be associated with the relative availability of the compound at the site of action. A survey of pertinent data (3) indicates that the rapid decrease in activity that is observed beyond the maximum is related to a limiting water solubility. An appreciable solubility in water is necessary in order that a substance can be absorbed and transported to the site of action in concentrations capable of eliciting a pharmacological response. Clark (4, 5) has given detailed consideration to the nature of the reaction of chemicals on cells and has pointed out that the obvious approach to the problem is in the application of the methods of physical chemistry.

A consideration of the hypnotics illustrates the extent to which the physical and/or the chemical aspects of a series of compounds have been emphasized in the "interpretation" of the mode of action of a single pharmacological group. The action of hypnotics on cells has been variously interpreted as being related to: (*a*) the ratio of lipid to water solubilities [Overton (6) and Meyer (7, 8)]; (*b*) surface tension lowering or adsorption [Traube (9), Lillie (10), and Warburg (11, 12)]; (*c*) inhibition of oxidation [Quastel *et al.* (13, 14, 15), Jowett *et al.* (16, 17)]; (*d*) cell permeability [Höber (18), Lillie (10), and Winterstein (19, 20)]. In addition to the above, several other explanations for anesthesia and hypnotic action have been proposed by Henderson (21), Meyer (22), Clark (23), and Beecher (24). The reader is referred to Goodman & Gilman (25) for a concise statement on some of the general postulates.

It is well known that the physical and chemical properties of a compound are determined by the number, kind, and arrangement of the atoms within the molecule. Also, it is known that the common physical and chemical properties of a molecule determine the availability for absorption, the degree of concentration on cell surfaces, or following penetration, the degree of concentration within the cell. Accordingly, the possible importance of such properties as solubility, partition coefficients, dissociation constants, surface tension, degree of dissociation at a given pH, etc. become apparent. Recently evidence has accumulated to indicate the importance of other physico-chemical properties such as hydrogen bond formation, resonance, and others. Although the present state of our knowledge does not permit a precise interpretation of the physical properties resulting from a given organic structure, the modern theories of organic chemistry open a new approach to the general problem of correlation of cell-drug reactions. A number of recent publications illustrate the successful application of this method of approach (26, 27, 28). It should be emphasized that at times it may be more expedient to correlate pharmacological response in a given series of compounds with the chemical structure while at other times it may appear to be more profitable to give greater consideration to specific physical properties of a given series.

The physico-chemical properties of the cells where action occurs is a consideration of no less importance than the physico-chemical properties of the drug. It is somewhat controversial whether or not drugs act within the cell or exert their influence solely through ad-

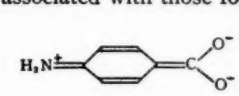
sorption on the cell surface. If the reaction occurs within the cell, then the process of sorption becomes an important factor.

Bacteriostatic and Bactericidal Agents

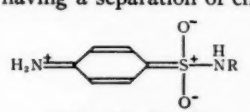
Sulfonamides (29, 30, 31).—The synthesis of new sulfonamide derivatives and the investigation of their antibacterial action continue to be the most active subjects in the field of synthetic drugs. Among recent reviews on the sulfonamides should be mentioned those of Marshall (32, 33), Dubos (34), MacLeod (35), and Schnitker (36).

From the standpoint of biochemistry, the most interesting development in this class of compounds is the elucidation of a mechanism that allows one to correlate the activities of these compounds with their physico-chemical properties. Earlier postulates (37 to 41) relating to the mechanism of action have been more or less superseded by the theory of Woods (42) and Fildes (43) wherein it is proposed that sulfanilamide exerts its bacteriostatic action by competing with *p*-aminobenzoic acid (PAB) for an enzyme system that is essential for the growth of the susceptible organisms. PAB is regarded as an "essential metabolite" that is synthesized by certain bacteria and is required in an enzymic system, or systems necessary for the normal metabolism of the cell. The close structural relationship of sulfanilamide and PAB is cited as a possible explanation for such a competitive mechanism. Subsequent investigations (44 to 53) have confirmed the essential character of PAB and have established that the bacteriostasis resulting from the sulfonamides is due to a competitive replacement of PAB. Ansbacher (54) and Martin & Ansbacher (55) indicate that PAB functions not only in the metabolism of bacteria and yeasts but in the metabolism of the higher animals as well. Green & Bielschowsky (45, 56) suggest that only the bacteriostatic substances antagonized by PAB should be considered to possess a true sulfonamide mode of action. They have established that compounds containing a *p*-amino group and bivalent sulfur are antagonized by PAB and appear to have a similar mode of action. When the aromatic amino group of a sulfonamide drug is oxidized (hydroxylamino or nitro), the oxidized form is no longer antagonized by PAB. From this it appears that the oxidation products of sulfanilamide have a different mode of action. Reduction products have a mode of action similar to that of the parent compound since they are antagonized by PAB. This evidence supports and strengthens the theory of Woods (42) and of Fildes (43). Additional evidence in support of this

postulate is reported by Wyss (57) and Wood (48) in which equations derived theoretically, relating the ionic concentrations of the sulfonamides and PAB, show the same linear relationship as that observed experimentally. Wood (48) studied the effect of PAB in inhibiting the bacteriostatic action of six sulfonamide drugs. In all cases the inhibition was proportional to the bacteriostatic power of the sulfa drug. Inhibition occurred when the concentration of PAB exceeded the concentration of the drug by a certain ratio. Within wide limits this ratio was an approximate constant for each drug. Bradbury & Jordan (58), making use of suspensions of *E. coli,* studied the electrokinetic mobility of the organisms in dilute solutions of PAB, sulfanilamide, and related compounds. All of the active sulfonamides had an effect on the mobility of the organisms resembling that of PAB. In the case of inactive substances related to sulfanilamide the effect is quite different. This observation is of considerable interest in that it indicates that the active sulfonamides behave like PAB at the bacterial surface. From comparisons of the effects on the electrokinetic mobility of sulfanilamide, benzenesulfonamide, and aniline hydrochloride, the authors conclude that the association of the drug with the organism is a function of the aromatic amino group and that the polarity produced by resonance is one of the factors that govern the bacteriostatic effect. Green & Bielschowsky (56) also find that association of the sulfonamides with the organism is a function of the aromatic amino group. According to the theory of Woods and of Fildes, the more closely the bacteriostatic agent resembles the "essential metabolite," PAB, the greater should be its activity. Kumler & Halverstadt (59) have pointed out that PAB and sulfanilamide have similar resonating forms with a separation of charge. They have suggested that the therapeutic effect of sulfanilamide type compounds may be associated with those forms having a separation of charge.



p-aminobenzoic acid



N^1-R-sulfanilamide

The *meta* isomers do not exist in such forms. This fact may have some bearing on their lack of activity. While the *ortho* isomers can exist with similar separation of charge, they may be rendered inactive through hydrogen bond formation or through a difference of spatial configuration.

A number of workers (26, 28, 60, 61) have correlated the activity of the sulfonamides with their dissociation constants as acids and bases. The results of such studies support the thesis that the activity of this type of compound is a function of the cationic or anionic form of the molecule. Bell & Roblin (26) have determined the acid and base dissociation constants of more than one hundred sulfonamides and related compounds. PAB has a K_b of approximately 2.3 to 2.6×10^{-12}. The range of basic strength of the aromatic *p*-amino group of the sulfonamides was found to be small (0.5 to 2.3×10^{-12}). While the base constants may be an important factor (61), the small variations in strength do not permit the correlation of this constant with the bacteriostatic activity. The acid dissociation constants, however, vary over a wide range (10^{-11} to 10^{-3}) and have been presented (28, 60) as evidence that the ionic form of the sulfonamides is chiefly responsible for the bacteriostatic activity. It is suggested by Fox & Rose (60) that the ions of different sulfonamide derivatives are equally active; the difference in the concentrations of the ionic forms parallels the difference in activity. The pK_a values of sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine are 10.5, 8.5, 6.8, and 6.4, respectively, and the per cent ionized at pH 7 is, in the order named, 0.03, 3.4, 61.6, and 80.0. Bell & Roblin (26), on the basis of sound experimental evidence, propose a theory relating structure to bacteriostatic activity. They observed that by plotting acid constants versus the activity *in vitro,* a curve is obtained which passes through a maximum as the acid strength increases. This correlation is shown to be directly associated with the negative character of the sulfonyl group; the more negative this group, the greater is the bacteriostatic effect of the sulfonamide. The negativity of this group is related to the electron-attracting power of the N^1-substituent. If the relative electron-attracting power is known it is possible to predict the bacteriostatic power of any new sulfonamide of this type.

The acid constants afford an indirect measure of the relative electro-negative character of the substituent group (R), since the change in acid strength is proportional to the change in the electronegativity of the substituent group. In order to be acid-strengthening, the substituent group must be an electron-attracting (electronegative) group. Using Branch & Calvin's (62) inductive constants for various N^1 substituent groups, it was found (26) that the I_R (inductive constant) is a linear function of the pK_a values of the corresponding sulfonamide. An equation was derived to determine the optimum pK_a that a sul-

fonamide drug should have in order to give maximum activity at pH 7. This was shown to be a pK_a of 6.7 and agrees satisfactorily with the experimentally observed maximum. This is a most important implication, based on both experimental and theoretical considerations, since it appears that the maximum activity of N^1-substituted sulfonamides has been attained. However, as pointed out by the authors and also by Schmelkes *et al.* (28), other properties, such as absorption, excretion, toxicity to the host, etc., are, from the practical standpoint, equally as important as the activity determined *in vitro*.

The evidence that association with the organisms is a function of the aromatic amino group (56, 58, 61), that the bactericidal activity is a function of the acid dissociation constant or the negativity of the sulfonyl group (26, 28, 60), and that the forms with a separation of charge are responsible for the activity (58, 63) may seem irreconcilable. Such, however, is not the case. The more negative the sulfonyl group, the more contribution will be made by the form with a separation of charge (i.e., the greater the positive charge on the *p*-amino nitrogen). This is similar to the case of *p*-nitroaniline (64). As the form with the separation of charge becomes more important, the amino group assumes a more coplanar configuration with respect to the ring. Kumler (63) suggests that this coplanarity of the amino group may be a necessary condition for reactivity with some enzyme system. The amino group in PAB is in all probability nearly coplanar with the ring. The antibactericidal activity of a number of other compounds including the *p*-amino sulfones, the ring substituted sulfanilimido dihydropyridines of Shepherd *et al.* (65), the monoamino acridines, and the triphenyl methane dyes may be interpreted on the same basis. Some of these are discussed in detail in the section on acridines. Shepherd *et al.* (65) found that the ring nitrogen methyl derivatives of sulfapyridine and sulfathiazole are less active than the parent compounds while the N^1-methyl derivatives are practically inactive.

That sulfonamide-inhibiting substances other than PAB are present in tissue and extracts of natural origin is well known. Loomis, Hubbard & Neter (66), Harris & Kohn (67), and Tsuchiya *et al.* (68) have shown that methionine exerts such an action. The latter investigators (68) made the interesting observation that urea completely neutralizes the inhibitory action of methionine on the sulfonamides and thereby enhances the bacteriostatic action of these substances. Recent investigations (69, 70) show that low concentrations of urea, while in themselves having no effect on the growth rate, re-

duce the concentrations of sulfonamides that are required to overcome the protective action of PAB. Schmelkes & Wyss (71) and Neter (72) have shown that azochloramid increases the bactericidal activity of the sulfonamides on cultures of *Staph. aureus* and *E. coli*. It is suggested that this potentiation may possibly be due to the inactivation of sulfonamide inhibitors. Snell & Mitchell (73) have demonstrated that certain of the pyrimidine bases, PAB, *dl*-methionine, adenine, guanine, xanthine, and hypoxanthine are growth-stimulating for several lactic acid bacteria. All of these substances in the presence of suboptimal quantities of PAB are capable of reversing sulfanilamide bacteriostasis for the same lactic acid-producing organisms. McIlwain and others (74 to 81 incl.) have approached the problem of bacteriostasis through a study of the inhibition of "essential metabolite" analogues. This is, of course, an extension of the concept developed by Woods (42) and by Fildes (43) and is designed to establish the extent to which the principle of inhibition applies. These investigators have shown that the growth-stimulating effect of nicotinic, aminocarboxylic, and pantothenic acids is inhibited by the sulfonic acid analogues of these acids; that indole-3-acrylic and barbituric acids likewise inhibit the growth-stimulating effect of tryptophane and uracil, respectively. These substances apparently act as inhibitors by virtue of their structural relationship to the essential metabolites. McIlwain (76) proposes to call the ratio C_1/C_M the "bacterial index" where C_1 is equal to the concentration of an inhibitor just bacteriostatic in the presence of a concentration of the corresponding metabolite, C_M. Such values, although probably subject to considerable variation, may find clinical application in specific infections. The index, as might be expected, varies with different organisms (76, 79). It has been suggested (56) that the sensitivity of bacteria to sulfonamides may be determined by the rate at which PAB is synthesized in the bacterial cell and/or is released into the surrounding medium. Rose & Fox (82) present evidence in support of this suggestion. The ratio, PAB/drug, was observed to be inversely proportional to the minimum effective concentration of each of the sulfonamides studied. This indicates that the bacteriostatic activity is related directly to the quantity of the drug required to produce complete inhibition in the presence of a given amount of PAB. Similar results are reported by Wyss and collaborators (83).

Keltch *et al.* (83a) have shown that seven local anesthetics derived from PAB exert an ***in vitro*** anti-sulfonamide effect. Nine local

anesthetics derived from other sources exhibited no antagonism to the sulfa drugs.

Antibacterial surface-active agents.—In 1935, Domagk (84), shortly after having reported on the antibacterial effect of prontosil (85), the parent compound of the sulfonamides, announced "a new class of disinfectants." This report concerned the bactericidal activity of the quaternary ammonium salts, which have since been variously called "cationic surface active agents," "invert soaps," "bactericidal detergents," etc. The announcement of the bactericidal properties of a new chemical class of compounds served to stimulate the interest of a great many workers in the field. The intermediate and higher molecular weight quaternary ammonium salts belong to one of three general classes of detergents in wide use: (*a*) Anion-active (i.e., ordinary soaps, salts of the bile acids, salts of the sulfate, or phosphate esters of the alcohols, and salts of sulfonated esters); (*b*) Cation-active (i.e., substituted ammonium, quaternary ammonium, sulfonium, and phosphonium salts); and, (*c*) Nonelectrolytic (polyglycol, or glycerol ester of fatty acids). The first two classes of detergents are colloidal electrolytes, as defined by McBain (86, 87), in which the hydrophobic ion forms a "colloidal particle or micelle by association of ions or ion pairs" to give a negatively or positively charged colloid ion. In the anionic type the anion is surface active and in the cationic type the cation is surface active. Many of the cationic type ("invert soaps") have been shown to exert a powerful bacteriostatic effect. The anionic type are usually much less active.

Baker, Harrison & Miller (88), Dubos (34), Klarmann (89), and Gershenfeld *et al.* (90, 91) have published reviews on various aspects of the surface-active bacteriostatic agents. Miller & Baker (92) and Baker, Harrison & Miller (88) have studied the action of various surface agents on the metabolism of bacteria. All of the cationic agents studied were found to be active inhibitors of the metabolism of both Gram-positive and Gram-negative organisms. The anionic agents were effective only against Gram-positive organisms. A number of investigators (88, 90 to 94) have shown the importance of hydrogen ion concentration on the bacteriostatic properties of wetting agents. Scales & Kemp (94) observed that at pH 4 anionic detergents were equally active against Gram-negative and Gram-positive organisms. At a higher pH, Dubos (95) and Baker and co-workers (88) report that the anionic detergents show inhibitor specificity for the Gram-positive organisms. Ordal & Borg (96) studied the effect of an anionic

surface-active agent and a cationic agent on inhibition of the oxidation of lactate by a Gram-positive and a Gram-negative organism. Both the anionic and cationic surface-active agents inhibited the oxidation of lactate by the Gram-positive *Staph. aureus*. The anionic agent did not inhibit the oxidation of lactate by the Gram-negative *E. coli*. Dubos (95) has pointed out that Gram-negative bacteria contain lipopolysaccharide antigens that appear to form an outer coating on the cells. Baker *et al.* (97) and Gershenfeld & Ibsen (93) have shown the protective action of certain phospholipids against the action of both cationic and anionic detergents. It is suggested by Ordal & Borg that a cell membrane containing substances of this character in the Gram-negative bacteria may explain their lack of sensitivity to the anionic detergents. Gershenfeld and collaborators (90, 91) and Baker *et al.* (88) have shown that the cationic detergents exhibit maximum bactericidal activity in the alkaline pH range while the anionic detergents are most effective in the acid range. It is of interest that the same observation was made by Stearn & Stearn (98, 99, 100) on the bactericidal activity of cationic and anionic dyes.

Extensive studies have been made in several laboratories on the influence of wetting agents on the activity of many commonly used antiseptics (90, 101, 102, 103). Gershenfeld & Perlstein (90) obtained a marked increase in germicidal efficiency for such mixtures except for those antiseptics that were chemically incompatible with the wetting agent. The activity of the mixture was greatly influenced by the hydrogen ion level of the solution under test. Ordal, Wilson & Borg (103) studied the effect of several soaps alone and in combination with sodium lauryl sulfonate on the bactericidal action of phenolic compounds. They observed increased germicidal activity. Fisher (102), working with approximately neutral solutions of phenols with cationic and anionic wetting agents, observed little or no increase in effectiveness. It is well established that phenolic compounds are usually more effective bactericides in acid solutions, and it is possible that increased activity might have been obtained if stronger acid solutions had been employed (90).

Kuhn and associates (104 to 111) have prepared and studied a large number of cationic surface-active compounds including quaternary ammonium, sulfonium, triazolium, and tetrazolium salts. They have also prepared and studied a series of azinium salts. The sulfonium compounds examined were qualitatively the same as the ammonium salts but were only one third as active (105). For each class

of compounds studied, Kuhn and associates find that in general the bactericidal action increases with increase in the length of the alkyl chain. The optimum length of the alkyl chain for maximum effectiveness may vary for each class; e.g., in the azinium salts the maximum bactericidal effect for *Staph. aureus* is given by the C_{12} chain while the C_8 and C_{16} salts are only 8 per cent as effective. Baker, Harrison & Miller (88), in studying homologous series of straight chain alkyl sulfates and sulfoacetates (C_8 to C_{18}), observed maximum effectiveness for the C_{12}, C_{14}, and C_{16} compounds. They made the extremely interesting observation that the lauryl esters of amino acids are active inhibitors, thereby demonstrating that the quaternary ammonium structure is not essential for bactericidal activity. This has also been shown by Westphal (110) who observed that mono-, di-, and tri-alkylamines, hydrazine, and azinium salts having one long alkyl group may show almost the same bactericidal activity as the quaternary ammonium salts. Baker, Harrison & Miller (112) observed an apparent relationship between chemical configuration and bactericidal effectiveness in that certain of the cationic detergents ("Phemerol" and "Zephiran") were considerably more active than other closely related compounds. Both compounds have a benzyl and two methyl groups attached to the nitrogen and differ only in the long nonpolar group. They suggest the possibility of the benzyl group specifically enhancing activity. The presence of the quaternary nitrogen in a ring structure does not appear to increase the bactericidal properties over those of the acyclic quaternary nitrogen derivatives.

There is comparatively little known concerning the mode of action of the surface-active compounds. Kuhn, Bielig & Dann (104) have observed that germicidal concentrations of some surface-active agents are comparable to the concentrations necessary to produce denaturation of proteins. This is not in agreement with the observations of Baker *et al.* (97) who find that the concentrations of detergents necessary for protein denaturation [as determined by Anson (113)] do not correspond to the concentrations necessary to affect the metabolism and viability of microorganizms. Moreover, the anionic detergents are active protein denaturants and yet show greatest activity for Gram-positive organisms. These observations have led Baker and associates (97) to the conclusion that one or more factors besides denaturation of proteins must be involved in the bactericidal activity of anionic detergents. They suggest, as a possible explanation of the rapid action of surface-active agents, that the denaturation

of proteins essential for metabolism is preceded by a disorientation of the cell membrane. Such a view does not necessarily involve a specificity for any particular group in the bacterial cell and may explain the increase in activity observed for various bacteriostatic and bactericidal agents in the presence of surface-active compounds. The surface-active agent may conceivably disorient the cell surface and thereby render the organism more susceptible to attack by other chemical agents.

The role of surface tension in the mechanism of action for the surface-active compounds appears to be of a secondary nature (90, 92). This has also been reported for another class of bactericidal agents (114). Valko & DuBois (115) have compared the behavior of surface-active cations to the action of other types of toxic cations such as the heavy metals and the basic dyes. They have observed the same reversibility of the bactericidal effect as that previously reported for mercury compounds (116 to 122) and the basic dyes (123). Under suitable conditions they were able to reverse the "killing" action of surface-active cations by detoxication with high molecular weight anions. Gershenfeld & Ibsen (93) and Baker *et al.* (97) have studied the effect of lipids and phospholipids on the inhibition of bacterial metabolism caused by surface-active anionic and cationic wetting agents. Interference of the normal inhibition on metabolism was also observed. Baker and associates (97) find that the phospholipids must be added first or simultaneously with the detergent to obtain interference. Addition after the detergent is without effect. In general, they found that germicidal quantities of the detergents are not effective in the presence of phospholipids but they did not observe a reversal of the bactericidal effect as reported by Valko & DuBois (115). The reversibility of the bactericidal action of mercuric chloride has been known for many years and was studied by Engelhardt (121), McCalla (116 to 120), and Fildes (122). Fildes (122) has shown that mercuric ions act on bacterial cells by combining with sulfhydryl groups. The "killing" action is reversible, even after long periods of time, by addition of sulfhydryl compounds which form soluble mercury derivatives (122), or, by addition of hydrogen sulfide (116 to 121) which forms the insoluble sulfide. Valko & DuBois (115) interpret the behavior of the surface-active cations as another example of the general phenomenon of ionic exchange. Stearn & Stearn (98, 99, 100) explained the antibacterial action of cations, including those of the heavy metals and basic dyes on the basis of reversible adsorp-

tion by the acidic groups of the bacterial cell surface. The inhibiting effect of basic and acid dyes may be reversed by changes in hydrogen ion concentration of the medium. The anionic dyes were, like the surface-active anionic substances, most active in acid solutions, and the cationic dyes most active in basic solutions. McCalla (120) has demonstrated the rapid exchange of a large number of cations by bacteria. Peck (124) has shown that the proteolytic action of trypsin is inhibited by soaps and that addition of calcium salts results in a reversal of the inhibition. DuBois & Valko (125) have studied the antibacterial effect of mixed surface-active cations. The data indicate that the effect of surface-active non-toxic cations on the antibacterial action of surface-active toxic cations is to lower the activity of the toxic ions ("subtractive"). It is assumed that the adsorption equilibrium of the toxic ions by the bacteria is shifted by virtue of the competition of the non-toxic cations for the same spaces on the bacterial surface. The antibacterial activity is believed by DuBois & Valko (125) to be determined by the affinity of the cation for the bacteria and by the specific toxicity of the cation *per se*. Hoffman, Schweitzer & Dalby (126) have observed the same type of weakening ("subtractive") effect by a weak fungistatic fatty acid on a strongly fungistatic acid. DuBois & Valko (125) interpret the effect of hydrogen ion concentration on the activity of the surface-active cationic and anionic wetting agents as resulting from a competition between relatively harmless hydrogen or hydroxyl ions and toxic ions of the same charge, for the active groups involved on the bacterial surface.

The ion exchange interpretation although supported with considerable experimental evidence assigns no importance to the undissociated molecule from which the ion is derived. In the case of weak acids and weak bases of low molecular weight, the bactericidal activity increases with change of pH in the direction of increasing concentration of the undissociated molecule which in turn is accompanied by an increase in lipid solubility, partition coefficient, etc. (114, 127, 128, 129). This is usually explained on the basis that the undissociated molecules are more readily available for adsorption on, and diffusion through, the cell membrane of the organism. The ion exchange explanation for the mechanism of action of bactericidal ions does not require ion permeability of the cell membrane. The fact that many active substances do penetrate is well known. Kuhn & Jerchel (109) have observed the apparent penetration of tetrazoliun salts into yeast cells and bacteria.

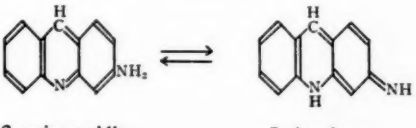
Findlay (80) observes that once an active chemical agent has been brought in the environs of a bacterial cell three stages are distinguishable. These are adsorption, interference with metabolism, and death, or such injury to the parasite that it is destroyed by the phagocytes of the host. When the agent becomes attached, it may act by preventing an essential food factor from being adsorbed, or by causing a breakdown in metabolism through combining with a specific substrate, or by competing with an essential cell metabolite for an enzyme or coenzyme. A single break in the chain of metabolic reactions may give rise to others. McIlwain (80, 123) has observed that when an organism is deprived of the use of enzymes or metabolites by various types of interference it becomes nutritionally more exacting than in its normal state and may require material not normally required when interference is absent.

Acridine dyes and related compounds.—Browning (130) first introduced the well known diaminoacridines as bactericidal agents. Browning *et al.* (131) later demonstrated that the bactericidal activity of this type of compound is due, in part, to the acridine nucleus; the analogues derived from quinoline and pyridine were shown to be inactive. It was observed that maximum activity occurred when amino groups were substituted in the acridine nucleus. Berry (132) has recently confirmed the finding that the intact acridine nucleus is necessary for bacteriostatic activity. Albert *et al.* (133) studied a series of ten diaminoacridines and correlated their activities with the positions held by the amino groups. Certain predictions were made for the activities of the monoaminoacridines. The validity of these predictions is confirmed by the work of Albert, Rubbo & Goldacre (134) and Rubbo, Albert & Maxwell (27) who studied the influence of positional isomerism on the bactericidal activity of the monoaminoacridines. It was observed that the bactericidal activity closely parallels the relative basicity of the various isomers. As suggested by the authors (27), the series may be divided into three distinctive groups of compounds.

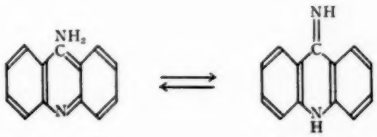
The first group, characterized by the presence of the imino grouping, shows the highest bactericidal activity and possesses the greatest basicity. The 2- and 5-monoaminoacridines, as well as proflavin, are assigned to this group. The second group, characterized by the presence of the normal amino group, shows moderate activity and also moderate basicity. The 3- and 4-monoaminoacridines are placed in this group. The third group has no bactericidal activity and shows

abnormally weak basic properties. The weak basic properties of the sole member of this group, 1-aminoacridine, are to be attributed to the fact that the amino group is masked through hydrogen bond formation.

GROUP I

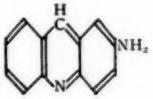


2-aminoacridine Imino form

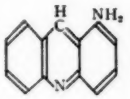


5-aminoacridine Imino form

GROUP II

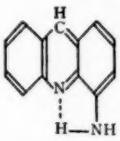


3-aminoacridine



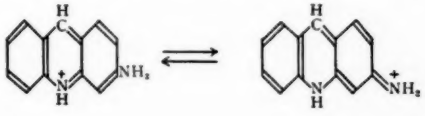
4-aminoacridine

GROUP III



1-aminoacridine

A study of the partition coefficient (oil/water) of the series brings out an additional trend. The activity increases with increase in water solubility (decrease in partition coefficient). This is suggestive of the fact that activity is to be associated with the ionic form of the molecule. These investigators suggest that the activity may be due to the basicity or to the presence of the imino structure. These two explanations are not independent. Thus the imino compounds are strong bases due to the fact that the ion has two resonating forms which lower the energy of the ion and make the base strong.



The activity appears to be more closely related to the structure associated with the imino ion than to the basicity of the compound. This type of structure is present in the sulfonamides (58, 59), in the triphenylmethane dyes (27), in the diaminophenoxathiins (135), and in the chloroaminoacridines (136). The structure common to all of these is one in which the amino group tends to assume a coplanar configuration. Kumler (63) has suggested that the coplanar amino group may be a necessary condition for reactivity with some enzymic systems and this is also proposed as a possible explanation for the activity of the sulfonamides. Irie (135) prepared a series of diaminophenoxathiins and observed bactericidal activity in the 3,6,- and 2,4-diamino compounds but not in the 2,7-isomer. Bradbury & Linnell (136, 137) have prepared and studied two series of chloroaminoacridines and observed that none of the chloro derivatives is as active as the parent amino compounds. Of the compounds studied, the 6-chloro-2-amino and 7-chloro-2-amino acridines are most active. The 8- and 9-chloro-2-amino derivatives showed no activity. These observations of Irie (135) and of Bradbury & Linnell (136, 137) are also in agreement with the suggestion of Kumler (63). Thus chlorine substituted in the 8- or 9- position of the aminoacridines will make possible new resonating forms which tend to reduce the contribution of the form having the coplanar amino configuration.

Local Anesthetics

This subject is deserving of a complete review from the standpoint of physical and chemical properties in relation to pharmacological activity. This might be treated in a manner similar to that employed for the bactericidal and bacteriostatic agents. However, because of limitations of space, such a method of treatment is not possible. With minor exceptions, this portion of the review will be restricted to a listing of some of the new compounds, together with references that appear to be of interest. Most of the recent publications are concerned with work on compounds closely related chemically to well known local anesthetics. A number of comparatively recent reviews on the pharmacology of the general and local anesthetics have been published (138, 139, 140). For a satisfactory general discussion the reader is referred to Goodman & Gilman (25).

p-Aminobenzoic acid derivatives.—Monoalkylaminoalkyl esters of *p*-aminobenzoic acid, first studied by Goldberg and associates (141, 142), have recently been studied by Kremer & Waldman (143).

Schamp *et al.* (144) have reported the results of a pharmacological study of 2-isobutyl-aminoethyl-*p*-aminobenzoate ("Monocaine"). The report indicates that the anesthetic efficiency and toxicity of this monoalkylamine derivative are similar to procaine, except that the cardiovascular paralyzing action and irritant effects are greater. These investigators were not able to substantiate current claims that the monoalkyl derivative has pressor activity, or that it enhances the action of epinephrine. Moore & Volwiler (145) prepared a number of dialkylaminoalkyl esters of mono- and dibromo-*p*-aminobenzoic acids. All of the monobrominated derivatives are efficient anesthetics and some have a favorable therapeutic index. However, the hydrochlorides of the ester bases are too insoluble in water to make useful anesthetics.

Alkylthio- and alkoxybenzoic acid derivatives.—The substitution of an alkoxy group for the aromatic amino group in aminoalkyl esters of *p*-aminobenzoic acid has been shown to give useful anesthetics (146). β-Diethyl-aminoethyl-*p*-ethoxybenzoate ("Intracaine") is representative of this. Pierce *et al.* (147, 148) recently prepared a number of β-monoalkylaminoalkyl esters of *o-*, *m-*, and *p*-alkoxybenzoic acids. Some of the compounds studied were strong local anesthetics. Rohmann, Haas & Bergstermann (149) have prepared a number of β-piperidinoethyl esters of *p*-alkoxybenzoic acids. The lower homologues correspond in anesthetic activity to procaine, while the higher members show an increasing activity up to four times that of procaine. The latter suffer from the disadvantage of being tissue irritants. Donleavy & English (150) have prepared a number of aminoalkyl esters of *o-*, *m-*, and *p*-alkylthiobenzoic acids. All of the compounds are reported to be less toxic than procaine and many of them give anesthesia of longer duration. It is of interest that the *o-* and *m*-ethylthio- and methylthio-derivatives are more active than the corresponding para-isomers.

Cinnamic acid derivatives. — Alkamine esters of cinnamic acid ("Apothesine" type), *p*-ethoxycinnamic acid (151), α- and β-alkylcinnamic acid (152, 153) are all reported to be active anesthetics. Fosdick & Starke (154) have prepared a number of alkamine esters of 4-acetylferulic and 3,4-dimethoxycinnamic acid. A pharmacological examination of the first member of each series indicates an activity slightly greater than procaine, but the compounds are relatively toxic.

Aminophthalic acid derivatives.—Blicke *et al.* (155, 156, 157) have studied three series of alkyl, dialkylaminoalkyl-aminophthalates: (*a*) 1-alkyl 2-dialkylaminoalkyl 3-aminophthalates; (*b*) 1-dialkyl-

aminoalkyl 2-alkyl 3-aminophthalates; and, (*c*) 1-alkyl 2-dialkylaminoalkyl 4-aminophthalates. Many of the compounds show strong local anesthetic activity. The 4-aminophthalate esters were inferior to the corresponding 3-amino derivatives.

Aminonaphthoic acid derivatives.—Blicke and associates (158, 159) and Sergievskaya & Preobrazhenskaya (160) have prepared a large number of mono- and dialkylaminoalkyl esters and amides (159) of aminonaphthoic acids. The esters show marked anesthetic activity while the amides appear to be inferior. The β-diethylaminoethyl ester of 4-aminonaphthoic acid ("Naphthocaine") is a more effective anesthetic agent than cocaine (161).

Tetrahydronaphthalene derivatives.—Starting with the work of Bamberger & Müller (162), a comparatively large number of tetrahydronaphthalene derivatives have been prepared and studied pharmacologically. A number of the compounds examined are reported to have anesthetic activity (163, 164, 165). Cook and Hill (166, 167) have prepared the benzoic and *p*-aminobenzoic acid esters and the phenylurethane derivatives of 2-alkylamino-3-hydroxy-1,2,3,4-tetrahydronaphthalenes. Several of the esters and the phenylurethane derivatives are active local anesthetics. It is also shown that the corresponding derivatives of 1-dialkylamino-2-hydroxy-1,2,3,4-tetrahydronaphthalene have local anesthetic properties (167).

Biphenyl derivatives.—Case & Koft (168) continued a study of the biphenyl analogues of procaine as initiated by Braker & Christiansen (169). The compounds are reported by Fellows (170) to be irritating to tissues, to show poor depth of anesthesia, and to have an unsatisfactory therapeutic index.

Heterocyclic compounds.—Because of the activity shown by esters of aminophthalic acid (155, 156, 157), Blicke & Jenner (171) prepared the corresponding esters of quinolinic acid in order to determine the effect of shifting the nitrogen into the ring. None of the esters containing nitrogen in the ring showed useful properties. The authors (171) also prepared alkamine esters of nicotinic acid, which had been previously reported by Ingersoll & Robbins (172). These esters were compared with the corresponding esters of *p*-aminobenzoic acid. β-Diethylaminoethyl nicotinate has high water solubility as compared to the solubility of active esters. This is suggested as a possible reason for its low anesthetic activity. Burtner & Lehmann (173) have prepared and examined a number of alkamine esters of carboxylic acid derivatives of carbazole, dibenzofuran and dibenzo-

thiophene. Active carbazole derivatives of low toxicity had been previously reported by Knoefel (174). β-Diethylaminoethyl 5-ethyl-carbazole-3-carboxylate, the most active member of the series, is reported (173) to be three times more active than cocaine and only one fifth as toxic. However, it is not regarded as a useful anesthetic because of its property of irritating tissues. Gaind, Ray & Sarin (175) have prepared carboxylic acid amide derivatives of carbazole some of which show strong surface anesthetic activity. Rose, Shonle & Chen (176) have studied a large number of derivatives of 2-aminobenzothiazole, 2-phenylamino-4,5-dihydrothiazole, 2-phenylamino-4,5-dihydroöxazole and 1-phenylimidazole. All are reported to be active local anesthetics and all but one produce surface anesthesia. Lur'e (177) in a study of alkamine esters of 2-alkoxy substituted cinchoninic acids, observed that the anesthetic activity depends somewhat on the 2-alkoxy group but chiefly on the ester group. All of the esters exert a powerful anesthetic action but most are irritating to tissues. Magidson *et al.* (178) have studied a number of alkamine amides of 2-alkoxy-substituted cinchoninic acid ("Percaine" type). In general there appears to be an increase in anesthetic activity and toxicity with an increase in the number of carbons in the alkoxy group. The increase in anesthetic power lags behind the increase in toxicity. The optimum length of the alkoxy group depends on the alkamine-amide group, changing of one requires a change of the other for maximum effectiveness.

Cook & Kreke (179) report on a study of alkylamine esters of furoic acid. This type of ester was first reported by Gilman & Pickens (180) and later by Phatak (181) and Phatak & Emerson (182). The esters in general have a low order of topical anesthetic activity but in certain instances are superior to the benzoic acid analogues.

Diphenylacetic acid and related derivatives.—The anesthetic activity of β-diethylaminoethyldiphenylacetate ("Trasentin") is well known. Gilman *et al.* (183) have studied the alkamine esters of diphenylacetic, diphenylpropionic, and benzilic acids, and of related acids. All of the esters are active local anesthetics. In most cases it was observed that toxicity and anesthetic activity is increased by increasing the length of the ester or alkamine groups. Alkamine esters of diphenylsuccinic acid are efficient anesthetics but give an exceedingly long duration of action.

Optically active compounds. — The enantiomorphic forms of a number of asymmetric local anesthetics have been pharmacologically

tested (184 to 188). Raasch & Brode (189) have examined the optically active phenylurethane anesthetics. Only slight differences in toxicity and anesthetic activity were observed for some of the optical isomers. Where differences exist the *l*-form appears to be least toxic and most active as an anesthetic. Schultz and Barbour (190) have reported on the anesthetic activity of *d*, *l* and *dl* cinnamylephedine. The various forms of this ester show a high and uniform activity. The *l*- form shows about one-half the toxicity of the *d*- form when tested on mice.

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PHOTOSYNTHESIS

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A survey of the literature published on photosynthesis covering the period 1939–1942[1] indicates a marked interest in the theoretical phases of the subject with special regard to the mechanism involved. Considerable attention also is given to environmental factors influencing carbon dioxide assimilation. On the whole, much activity has been directed toward methods and interpretation of data in a greater effort to understand this fundamental process of plants that is so essential to life. The problem has been attacked by the various well-known gaseous exchange methods, although more and more work is being done with tracer elements and the relation of fluorescence to the process. The experimental plants range from bacteria and algae to many of the higher plants. Also, leaf macerates and isolated chloroplasts have been studied. In order to keep within the allotted space, the authors have restricted this review to papers which appear to them to have a direct bearing on the problem of the general mechanism of photosynthesis. Many significant contributions to the ecology or physiology of various plants or plant groups are omitted of necessity. The trend of research is clearly toward the isolation of various parts of the photosynthetic mechanism. In this direction physical methods (intermittent light, induction effects, fluorescence, etc.) have been extended and chemical techniques have now become available (tracer elements, the Hill reaction). The older conception of a rigid and closely knit mechanism is giving way to the viewpoint that the photosynthetic processes are neither so utilized nor closely knit as supposed and that these processes have many steps identical with or similar to those of other metabolic reactions.

[1] Reference has been made to a few earlier papers which have important bearing on recent work. Literature citations to these papers have been omitted since they may be found in previous reviews of this series (1, 2).

Several general treatments of the subject have recently been published (3 to 7).

Steady-State Conditions

Effects of light intensity.—Sargent (8) has studied the effect of light intensity on the development of the photosynthetic mechanism. *Chlorella* cells grown under higher intensities show greater rates of photosynthesis at all light intensities and with carbon dioxide or cyanide concentrations used. The intensities at which maximum rates of photosynthesis are reached are considerably higher than the intensities used for cultivation. Unfortunately, the latter were so low as to be always light-limiting.

Inhibiting effects of high intensity (solarization) have been observed. Stålfelt (9) has found for two species of lichens a reversible light inhibition of photosynthesis. The inhibition is about 26 per cent of the average rate after ten hours exposure to 16,000 lux. Above 20° C. an additional irreversible temperature-inhibition occurs [cf. (10)]. At high intensities, up to 40,000 f.c., Myers & Burr (11) have observed a steady rate of oxygen uptake by *Chlorella,* greater than the rate of dark respiration and increasing with intensity. The various aspects of this phenomenon are explained in terms of a photooxidation following partial or complete inactivation of some part of the photosynthetic mechanism. Franck & French (12) have studied directly photooxidation in *Hydrangea* leaves in vessels containing alkali for removal of carbon dioxide. Photooxidation (oxygen uptake caused by light) is observed in living and dead leaves and in leaf macerates. Its dependence upon oxygen pressure is entirely different from that shown by dark respiration. It can be brought about by red or blue light and is thus presumably sensitized by chlorophyll. Photooxidation is pictured (12) as always taking place at high intensities, though at rates much lower than photosynthesis; only under carbon dioxide limitation or after any depletion of intermediates is some part of the photosynthetic mechanism destructively attacked.

Effects of wave length.—Eichhoff (13) has reported comparatively high rates of photosynthesis paralleling radiant energy absorption of a *Chlorella* suspension at various wave-length bands from 5150 Å to 8350 Å in the infrared. However, there is some question whether due consideration was given to the limitations of the Christiansen filters which he used. Noddack & Eichhoff (14), [cf. also (10, 13)] have measured photosynthetic rate as a function of intensity for white and

fairly monochromatic red light (~6400–6600 Å). Shapes of the light-saturation curves are different, being linear over a longer range and breaking more sharply to light saturation in the monochromatic light. Unfortunately, in no case is it clear that identical suspensions were used for obtaining the two curves.

Evidence has accrued for the participation of pigments other than chlorophyll in light absorption for photosynthesis. Montfort (15) has compared pigment absorption and photosynthesis yield in relatively broad bands isolated by filters in the blue and red. In diatoms and brown algae the yield is much higher than can be accounted for on the basis of chlorophyll absorption alone. Similarly, Mothes *et al.* (16) have reported that rates of growth of diatoms were actually greater in blue light than in red light of equal energy. Dutton & Manning (17) have found that the quantum yields of *Nitzschia* were equal in a broad red band (>6000 Å) and in the 4047–78, 4358, and 5461 Å mercury lines. The yield obtained in a narrow band at 4960 Å was somewhat lower. Absorption curves for the extracted pigments were recorded for chlorophyll, fucoxanthin, and other carotenoids. Even at 4960 Å it was thermodynamically impossible to account for the observed yield in terms of the energy absorbed by chlorophyll alone. Since the saturation rate of photosynthesis was found to be the same in red, blue, and mixed red and blue light, it seems that the energy absorbed by chlorophylls and other participating pigments is transferred to the same enzyme system [cf. also (10)]. By the use of narrow bands isolated by a grating monochromator, Emerson & Lewis (18) have similarly demonstrated in a most elegant manner the participation of phycocyanin along with chlorophyll in photosynthesis in the blue-green alga *Chroococcus*. Some degree of participation of the carotenoids was also indicated. [Other recent work has also appeared on the effects of light of different wave lengths (cf. 19, 20, 21).]

Effects of carbon dioxide, temperature, and other factors.—Livingston & Franck (22) have explored the effects of high carbon dioxide concentration on gas exchange in illuminated *Hydrangea* leaves. By conditioning the leaves with stepwise increases in carbon dioxide concentration they can be made to photosynthesize at relatively high concentrations of carbon dioxide. The rate falls roughly linearly with concentration of this gas. Half the maximum rate could still be obtained in 20 per cent carbon dioxide. By use of the Blackman palladium-black apparatus, Ballard (23) has observed a depression in rate of oxygen production by concentrations of carbon dioxide

in the range of 2 to 6 per cent. In both the above papers there is suggested a similarity between the effects of cyanide and high carbon dioxide concentration.

Noddack & Kopp (10) obtained data on *Chlorella* for the influence of temperature on photosynthesis and respiration and the influence of light intensity at various temperatures for white and monochromatic (~6400–6600 Å) light. They interpret a slight temperature coefficient at low intensities in terms of light-induced respiration.

Greenfield (24) has found certain differences between high and low intensities in the effects of certain salts on *Chlorella* photosynthesis. The relation of potassium deficiency to photosynthesis has been further investigated by Pirson (25). Potassium-deficient *Chlorella* shows a decreased photosynthesis which quickly responds to addition of potassium, rubidium, or cesium. These and other observed effects may be related, at least in part, to effects on permeability [cf. (2, 26)]. A salutary effect of ascorbic acid has been claimed (27).

Quantum Efficiency

The work of Warburg & Negelein (27a) in 1923 characterized the photosynthetic mechanism with an efficiency of about 0.25 molecules of carbon dioxide reduced per quantum of absorbed light. The thermodynamically calculated quantum requirement for the reduction of one molecule of carbon dioxide and evolution of one molecule of oxygen is about 0.34 (at 7000 Å). The comparatively high observed efficiency has far-reaching implications as to mechanism since so little energy can be available for the stabilization of intermediates. This vital question has been reinvestigated in a number of laboratories over the past five years with results which indicate a much lower efficiency.

The extensive work of the Wisconsin laboratory has led to values of from 0.04 to 0.11. Methods of measurement of photosynthesis have included gas-absorption analysis for carbon dioxide and Winkler method for oxygen [Manning *et al.* (28)], the dropping mercury electrode [Petering *et al.* (29) and Dutton & Manning (17)], and a photocalorimeter [Magee *et al.* (30)]. A number of different thermocouples were used in measuring radiant energy and these were calibrated against both standard lamps and the uranyl oxalate actinometer. Various methods of treatment of the plant material were used, yet the highest single value reported was a quantum yield of 0.11.

Careful repetition of the manometric technique and experimental procedure of Warburg & Negelein by Rieke (31) again led to a yield

of 0.25. Yet he noted that this high value depended upon selection of certain time intervals for calculation of rates of photosynthesis and respiration.[2]

Emerson & Lewis (32, 33) have systematically investigated the particular procedure of Warburg & Negelein and of Rieke. By refining and speeding up the manometric technique, Emerson & Lewis have been able to show that within the time intervals selected by Warburg & Negelein the CO_2/O_2 quotient is not constant but varies greatly due to a sudden "gush" of carbon dioxide on illumination and "gulp" on darkening. Changes in rate of oxygen exchange in light and darkness were much more regular. It was further shown that the sequence of readings, the temperature, carbon dioxide concentration, and conditioning of plant material were such as to enhance the carbon dioxide anomaly and increase its effect on the calculated quantum yield. By taking advantage of the previous conventions of calculation, a "yield" of 0.33 could be obtained. On the other hand, if errors due to the carbon dioxide were avoided, or if measurements were made in carbonate-bicarbonate buffer so that only oxygen was exchanged, then quantum yields were obtained which group around a value of 0.10. Similar yields have been reported by Rieke (31) for the same carbonate-bicarbonate buffer mixture.

Results at variance with those of Rieke and of Emerson & Lewis have been obtained by use of the carbonate-bicarbonate buffer mixtures. Eichhoff (13) has reported a number of values approximating 0.25 obtained over a range of wave lengths from 5150 to 7500 Å. At present no explanation of this discrepancy is apparent. However, the clear-cut explanation of the error in the technique of Warburg &

[2] The most difficult problem in efficiency studies is introduced by the fact that measurements must be made at low intensities where rates of respiration and photosynthesis are of the same order of magnitude. Respiration is conventionally measured in darkness and assumed to be equal in light. Yet there are frequent suggestions in the literature that under these conditions rate of respiration is not the same in light and in darkness, and large errors may be introduced by inaccurate estimation of respiration. In order to minimize such fluctuations Warburg & Negelein chose a sequence of alternating ten minute periods of light and darkness so that respiration might approximate a steady state with minimum deviations between light and darkness. Their calculations were then based on readings taken at the fifth and tenth minute of each dark period.

Furthermore, the technique depends upon the differential solubility of oxygen and carbon dioxide. In the experiments of Warburg & Negelein and of Rieke it was necessary to assume a value for the quotient of gas exchange for $CO_2/O_2 \sim 1.0$, as obtained under more favorable conditions of measurement.

Negelein, and the substantial agreement obtained in three different laboratories by a variety of techniques and on many types of plant material (33) point toward acceptance of a maximum quantum yield of 0.08 to 0.12. In the present state of our knowledge it is not important whether the maximum yield is 0.08 or 0.12. But it is of crucial importance whether the yield is high (~0.25) or low (0.08 to 0.11).

The Physical Nature of the Photosynthetic Apparatus

The extensive literature on the plant pigments has been reviewed by Mackinney (34) and Zscheile (35) and does not come within the present discussion except as to the question of the participation of the various pigments in photosynthesis.

The chlorophyll-protein complex and aqueous extracts of the chloroplast substance have been prepared and studied by various techniques (36 to 41). Evidence has been presented by Smith (37) that the chlorophyll is bound to a protein in a true chemical linkage. When dispersed by detergents, the pigment-protein complex showed sedimentation velocities which indicate a minimum molecular weight of about 265,000 (38). Neish (39) has presented data on the distribution of various materials between the chloroplast and the rest of the cell. Iron, and particularly copper, are concentrated in the chloroplast. Electrophoretic properties of the complex have been described by Fishman & Moyer (36). A lack of dependence of mobility upon particle size suggests that the complex is not altered during the fragmentation of the chloroplast.

Water extracts of the pigment-protein complex of various photosynthetic bacteria have been prepared by supersonic vibration [French (42)] and by grinding [Katz & Wassink (43)]. Various optical and chemical properties of the complex have been described. Extracts give no evidence of photosynthesis but will bring about photochemical oxidation of ascorbic acid (42). Wassink *et al.* (44) have compared the spectral absorption curves for suspensions of various photosynthetic bacteria. The alcoholic extracts of all strains show a single absorption maximum at 7740 Å. However, the living cells show two or more distinct maxima grouped at about 8000, 8500, and 8950 Å. The relative heights of these maxima differ between species and even in one species between culture conditions. It is logically suggested that in these bacteria a single bacteriochlorophyll is bound to different proteins, thus giving complexes with different absorption spectra.

At present there is little connection between the work on chloroplast (or cell) make-up and the kinetic data of photosynthesis. It may be expected that development and elaboration of the Hill reaction (*vide infra*) will lead to future correlation.

Intermittent Light

Emerson *et al.* (45) have studied rate of photosynthesis in relation to chlorophyll concentration in intermittent light (light flash, 10^{-5} sec.; dark period, 0.05 sec.) for *Chlorella pyrenoidosa*. The ratio of moles of chlorophyll to moles of carbon dioxide reduced per flash varied greatly (3,750 to 14,500) depending upon the age of the culture and growth conditions. This is at variance with earlier conceptions of an approximately numerically constant photosynthetic unit.

Weller & Franck (46) have repeated and confirmed the crucial experiments of Emerson & Arnold on flashing light. Light flashes (0.0045 sec.) were alternated with variable dark periods (0 to 0.129 sec.) by means of sectors. Curves for yield of photosynthesis per flash against length of dark period confirm the results of Emerson & Arnold. At 20° C. the curves approach hyperbolically the maximum yield per flash, indicating dependence on a first-order reaction. The maximum yield per flash is thought to be limited by a catalyst ("catalyst B") which stabilizes the products of the photochemical reaction and requires a recovery period identifiable with the length of the dark time (half time ~0.01 sec. at 20° C.). Cells poisoned by cyanide give a curve linear in shape at shorter values of the dark times and approaching asymtotically the maximum yield per flash of unpoisoned cells. This is interpreted to mean that the enzyme inactivated by cyanide is not the one responsible for saturation in the absence of inhibitors. The cyanide-sensitive catalyst (catalyst "A") is identified with the one effective in the fixation of carbon dioxide by an acceptor molecule [cf. Ruben *et al.* (47)]. The linear shape of the curve, obtained with organisms poisoned by cyanide, is explainable if this enzyme acts on a stable substrate. Behavior at low temperatures is explained in terms of the effects of the above two catalysts. The effect of hydroxylamine is explained in terms of a third catalyst ("catalyst C") controlling peroxide decomposition. Studies on longer flashes and dark periods in some cases gave the increased yields found for this type of experiment by Warburg and by McAlister. Weller & Franck identify such effects with the phenomenon of the dark pickup (48) and explain them in terms of limitations of "catalyst A."

Briggs (49) has discussed from a theoretical point of view some unpublished data on intermittent illumination with longer periods. Experimental details are not available and there are uncertainties in terminology (i.e., the term "yield"); however, it seems that the long-flash phenomena described by him are similar to those considered by Weller & Franck.

Kennedy (50) has compared (for two arbitrarily chosen dark periods) the oxygen evolution per flash per unit chlorophyll for magnesium-deficient versus normal and for heat-treated versus normal *Chlorella* cells. Using the conventional[3] reasoning, the data are interpreted to show that magnesium deficiency lowers the rate of the Blackman reaction and that heat-inactivation (45° C.) affects some other part of the mechanism.

Fluorescence

The probability of an excited clorophyll molecule losing its energy as fluorescence should depend upon the chemical environment of the chlorophyll. Earlier work was dictated by this theoretical consideration. Correlation of fluorescence intensity and photosynthetic activity has recently been accomplished.

Wassink *et al.* (51) studied the fluorescence intensity and rate of oxygen production by *Chlorella* under steady state conditions. The fluorescence yield (fluorescence intensity/incident intensity) was increased by urethane concentrations which partially inhibit, and cyanide concentrations which completely inhibit oxygen production. But fluorescence was not influenced by any of a number of conditions which markedly affect oxygen production (i.e., temperature, partial inhibition by cyanide, and oxygen pressure). Also, there was no change in fluorescence yield in passing from a light-limiting condition of photosynthesis to light saturation.

The work of Franck *et al.* (52) and McAlister & Myers (53) clearly indicates that for leaves the fluorescence yield increases for light intensities at or near those required for light saturation of photo-

[3] A tacit assumption in flashing-light work is that the limiting yield per flash is determined by the speed of a chemical reaction. It seems likely that in many cases this is a valid assumption, but it need not always be so. Physical limitations, such as permeability [cf. Gaffron (26)], may play an important role directly or indirectly though in this connection they have seldom been emphasized. Considerable danger lies in the simplification implied by the picture of a "light" and a "dark" reaction.

synthesis.[4] Effects of oxygen, cyanide, temperature, and carbon dioxide on the fluorescence yield can readily be demonstrated when appropriate plant material and light intensities are chosen. It would seem that the experiments of Wassink *et al.* were confined to a too limited range of conditions.

Much of the fluorescence work can best be considered in relation to the induction period. The very nature of fluorescence and the experimental work to date indicate that no simple relation is always to be expected between fluorescence intensity and rate of photosynthesis *in toto*. On the other hand, there is every reason to believe that fluorescence studies will prove more and more useful as it becomes possible to study various parts of the photosynthetic mechanism. Its measurement is not difficult when high speed of recording is not required [cf. (53)].

Induction Effects

When a green plant is illuminated with light of not too low intensity, there occurs an induction period (approximately 1 to 3 min.) before a steady rate of gas exchange or constant intensity of fluorescence is attained. Simultaneous observation of rate of oxygen production, rate of carbon dioxide uptake, and intensity of fluorescence have not yet been possible. Independent measurements by these three different methods show that induction behavior may be exceedingly complex and may show great variation, depending upon internal and external factors. Harmonious interpretation of all such data is therefore difficult.

Of particular importance in any theory of photosynthesis are two characteristics of induction whose validity has not been affected by recent work. The normal induction period is short, e.g., three minutes or less, even for algae which have been grown heterotrophically and have never previously been exposed to light (54); and its length, or any other quantitative measure of it, is greatest at high intensities and decreases to unmeasurably small values in weak light.

McAlister (48) found that a quantitative criterion better than length of induction period is induction loss, i.e., the additional amount of carbon dioxide that would be taken up if the carbon dioxide uptake

[4] It must be remembered that with leaves or with dense algal suspensions the cells contributing principally to the measured fluorescence may receive either a greater or lesser incident intensity (depending upon the experimental setup) than the bulk of the cells contributing to the measured gas exchange.

were to start at its final steady rate. A counterpart of the induction loss, a dark pickup, is observed as a briefly continued carbon dioxide uptake when the plant is suddenly darkened after intense illumination. The induction loss, dark pickup, and number of chlorophyll molecules are all of the same order of magnitude (48). This suggests that the chlorophyll molecules participate as individuals rather than as 2000-molecule aggregates as postulated from earlier flashing-light studies. That the dark pickup and induction loss are not merely the result of an extraneous reservoir would seem to be indicated by later work (53, 55, 56) showing that the time required for these phenomena is lengthened by cyanide and low carbon dioxide concentrations.

A rapid hot-wire conductivity method has been applied by Aufdemgarten (55, 57) to the measurement of the time course of carbon dioxide uptake for the alga *Stichococcus*. Because of the high time resolution of the method, it was possible to demonstrate a marked inflection, often a pronounced maximum and minimum, in the curves for rate of carbon dioxide uptake during the induction period. The magnitude of the inflection varied and may even have disappeared after certain culture conditions. Even after correcting his observed curves for instrumental lags, Aufdemgarten believes that a true inflection is indicated, i.e., that the rate of carbon dioxide uptake rises rapidly from an initial zero rate and goes through a maximum and minimum before reaching the final value.

By refinements in the infrared spectrographic method, McAlister & Myers (56) have been able to reproduce Aufdemgarten's carbon dioxide uptake curves with *Chlorella*. Again the inflection is dependent on culture conditions. A much less pronounced but possibly analogous effect is demonstrable with wheat seedlings. After correcting their observed curves for instrumental lags, McAlister & Myers interpreted the induction curves as indicating that the rate of carbon dioxide uptake is initially high, drops rapidly, and then rises more slowly to the end of the induction phase.

The study of the time course of fluorescence has been extended with particular attention to the effects of various factors known to influence photosynthetic activity. Kautsky & Eberlein (58) have improved upon Kautsky's earlier work by redesigning the fluorescence-measuring apparatus and illuminating only with wave lengths known to be photosynthetically active. They studied effects of oxygen pressure and temperature on the initial fluorescence behavior of *Ulva* during the first three of fifty seconds of illumination. Their postulation

of an oxygen-dissociable compound as acceptor for the activation energy of chlorophyll seems premature in view of the limited scope of their work. Wassink & Katz (59) have made similar studies on *Chlorella* over the first three minutes. They have worked on the effects of cyanide, temperature, and light intensity. The induction curves generally show two maxima occurring at about three and twenty seconds. They interpreted their curves in terms of a competition for the energy of excitation of chlorophyll between the normal acceptor and oxygen.

McAlister & Myers (56) have measured the fluorescence intensity simultaneously with the rate of carbon dioxide uptake for *Chlorella* and wheat. In such curves is to be found the best evidence yet presented for the validity of fluorescence measurements as a tool in the study of photosynthesis. Under certain conditions a clearly inverse relationship was observed between intensity of fluorescence and rate of carbon dioxide uptake. Under other conditions a direct relationship obtained. The behavior in respect to both fluorescence and carbon dioxide uptake was influenced by the previous history of the plant, carbon dioxide pressure, oxygen pressure, and light intensity. They believe that their observations can be explained in terms of the opposing processes of photosynthesis and photooxidation but do not suggest any details of the mechanism involved.

Franck *et al.* (52) have studied extensively the time course of fluorescence in leaves and algae. They also found a wide variety of behavior. Their data on the effects of inhibitors, temperature, carbon dioxide concentration, and light intensity are the most comprehensive yet obtained. They explain induction period in terms of the earlier hypothesis of Gaffron (2, 26) that a catalyst, oxidized and inactive in the dark, becomes progressively activated in light by the reducing action of photosynthesis. They identify this enzyme ("catalyst C") as the one responsible for the splitting off of oxygen from peroxides. Variations in induction behavior are explained in terms of the varying concentration of a metabolite and its effect on other catalysts when oxidized by peroxides accumulating during the induction period.

The various fluorescence induction curves (52, 56, 58, 59) aside from very general features, have little in common. This can be attributed to differences in time resolution of recording and to variations in the choice and condition of experimental material.

Gaffron (60) and Michels (61) have studied the effect of long anaerobiosis in the dark on subsequent behavior in light. In certain

strains of algae studied by Michels, photosynthesis was almost completely inhibited by such treatment. The inhibition was removed by illumination, by addition of some dehydrogenator such as quinone, or by suspension of the cells in alkaline media. The inhibitor is believed to be an acid product of fermentation although it could not be identified with any of the common organic acids. Other algae chosen by Gaffron were not so inhibited but showed abnormalities in induction behavior which could be related to their anaerobic dark metabolism. While extremely various, such types of behavior led Gaffron to suggest the general principle that "the enzymatic system in the plant which liberates molecular oxygen may also be able to transfer oxygen to hydrogen donors." This suggestion provides an explanation of the varying CO_2/O_2 quotients observed in Gaffron's extensive experiments. In spite of numerous claims that the quotient remains unity during the normal induction period [cf. (55, 60)], consideration of various induction data and the complexities of observed behavior suggest that this is not always the case. This is only another way of saying that rate of oxygen production and rate of carbon dioxide uptake may not show the same behavior during the induction period [cf. (54)].

Finally a word must be said in regard to the anomaly in gas exchange[5] observed by Emerson & Lewis (32, 33) in their quantum efficiency studies, *vide supra*. If the carbon dioxide burst results from the effect of light on a reservoir extraneous to the photosynthetic mechanism, then any interpretation (52, 56) of the induction behavior of fluorescence and carbon dioxide uptake in relation to photosynthesis is open to serious question. However, there is reasonable doubt as to whether there is any relation between these effects and those, for in-

[5] A word of caution should be recorded in regard to the quantitative interpretation of rapid manometric data such as these. Thermodynamic constants, obtained under equilibrium conditions, are used in the calculation of changes in rates. It must be assumed that rates of diffusion across the gas-liquid interface are equal for oxygen and carbon dioxide; the validity of this assumption has not been established. It must also be remembered that from the very nature of the gas concentrations usually prevailing, there may exist considerable differences in the possible diffusion gradients. Thus, during carbon dioxide uptake and oxygen evolution the carbon dioxide gradient is limited to its concentration (usually 5 per cent) while the oxygen gradient might correspond to a differential of 80 per cent under ordinary conditions. Rates of pressure change are no more than expressions of these diffusion gradients. Such considerations do not speak against the reality of the effect observed by Emerson & Lewis but only against the accuracy of its quantitative interpretation.

stance, of McAlister & Myers (53), in view of the extreme differences in conditions of light intensity and carbon dioxide pressure employed. Furthermore, it has not been demonstrated that the reservoir effect is really extraneous to the process of photosynthesis. Franck (62) has taken the view that it is not. The relation of this phenomenon to induction period is of critical importance.

Bacterial and Other Photosyntheses

Bacterial photosyntheses have been reviewed by van Niel (63). The case for van Niel's generalized equation of photosynthesis is strengthened by Foster's discovery (64) of an organism among the *Athiorhodaceae* for which an organic substrate fulfills exclusively the function of a hydrogen donor for photochemical reduction of carbon dioxide. In light this organism brings about the reaction

$$2\,CH_3CHOHCH_3 + CO_2 \rightarrow 2\,CH_3COCH_3 + (CH_2O) + H_2O.$$

A stoichiometric relation can be shown between the first three terms of the equation.

The quotient, H_2/CO_2, in the photosynthesis of *Streptococcus varians* under high light intensities has been shown by Wessler & French (65) to be about 2.6 rather than 2.0 as previously supposed. This suggests that the maximum quantum yield previously reported by French (2) for this reaction should be about 0.20 rather than 0.25.

The sigmoid nature of the light intensity curve reported by French (2) for *Streptococcus varians* has again been explained by Wohl (66) in terms of unstable intermediates. Other explanations of this phenomenon are possible (63, 65).

Direct evidence of a relationship between bacterial and green-plant photosynthesis has been obtained by Gaffron (3, 60, 67, 68) who "has achieved an experimental modification of the behavior of green algae so that photosynthetically they have become purple bacteria" (63, p. 287). When a small amount of oxygen was added to certain algae which had been maintained anaerobically under hydrogen for some time in the dark, an uptake of hydrogen and oxygen occurred. If carbon dioxide was present, it was also taken up and its reduction could be demonstrated (3, 68). This observation is a demonstration in a green plant cell of what has classically been known as chemosynthesis. Under low intensities of light the same algae, if previously maintained anaerobically under hydrogen and carbon dioxide in the dark, showed photoreduction of carbon dioxide and uptake of hydro-

gen similar to that of various purple bacteria (cf. 65). No oxygen evolution could be demonstrated under such conditions. But under higher intensities a transition took place from such photoreduction to normal photosynthesis with the usual oxygen evolution (67).

These findings logically lead to the conclusion that

> The difference between photoreduction and photosynthesis results, then, from the two ways by which the oxidized part of the photochemical oxido-reduction system is regenerated. It can be reduced either by hydrogen donors or by the removal of oxygen which finally appears as free gas (3, p. 254).

Thus, bacterial and green-plant photosynthesis would seem to share a common photochemical mechanism. The differences in experimentally determined quantum yields and in calculated energy requirements for the green plant and bacterial photosyntheses suggest that the former may require additional photochemical steps [cf. (3), p. 242].

Intermediates and Intermediate Reactions

New techniques have become available for the study of intermediates or of component reactions within the photosynthetic mechanism. In certain plants (e.g., sunflower) considerable amounts of carbon dioxide may be reversibly taken up as carbonate or bicarbonate of the sap (Smith, 69). The use of radioactive carbon (C^{11}) as a tracer by Ruben *et al.* (70) and by Smith & Cowie (71) has demonstrated a fixation of carbon dioxide in organic form by a reaction not requiring light. The light and dark assimilation of radioactive carbon dioxide has been extensively studied by Ruben and co-workers (47, 72, 73, 74). The uptake of radioactive carbon dioxide in *Chlorella* results in a water-soluble compound containing at least one alcoholic hydroxyl and one carboxyl group per molecule. This compound has a molecular weight of $\sim$1000 and a concentration of the same order of magnitude as chlorophyll. The dark uptake proceeds only to a limited extent and nearly all the radioactive carbon is localized in carboxyl groups. In light the uptake continues indefinitely at a constant rate and a smaller fraction of the total labeled carbon is located in carboxyl groups. Except for the difference in the location of radioactive carbon and a slight possible difference in diffusion coefficients (73), it is impossible to distinguish between the intermediates formed in light and darkness. Photosynthesis, as measured by either the manometric or radioactive method, and the dark uptake of labeled carbon dioxide show the same sensitivity to cyanide and ultraviolet

irradiation (2537 Å). In spite of repeated attempts, radioactive carbon could never be recovered as formaldehyde or other similar aldehydes of low molecular weight, or as acids. These results led to the postulation that "The RC*OOH formed in the dark is reduced to RC^*H_2OH in the light and the RC^*H_2OH takes on another carbon dioxide molecule" (73).

The brief note of Frenkel (75) further indicates that for *Nitella*, intact cells are necessary for the dark assimilation and that the compound in which labeled carbon is fixed in the dark is either nonchloroplastic or only weakly held by the chloroplasts. In light, radioactive carbon becomes so fixed in the chloroplast that it cannot be washed out with an isotonic aqueous solution.

Recent theories [cf. van Niel (63)] are corroborated in the experiments on heavy oxygen (O^{18}) by Ruben *et al.* (76). The oxygen evolved in photosynthesis originates solely from water. No indications could be obtained of exchange reactions involving molecular oxygen. An attempt (77) to investigate possible interconversion of chlorophylls *a* and *b* in photosynthesis by use of radioactive magnesium was not successful with the short-lived isotope available.

The production of oxygen by isolated chloroplasts has long been known as a transient phenomenon of small magnitude. Hill (78) has found means of increasing the rate and extent of this reaction. He was able to follow quantitatively the evolution of oxygen by spectroscopic measurement of the hemoglobin-oxyhemoglobin equilibrium. Fresh suspensions of chloroplasts on illumination evolve substantial amounts of oxygen in the presence of aqueous extracts of acetone-treated leaves. Carbon dioxide is apparently not taken up. Thus at once there is provided a means for studying a reaction similar to or identical with a part of the photosynthetic system of reactions, and a test for a probable intermediate in photosynthesis (79). Extracts of yeast, and subsequently ferric potassium oxalate, proved to be better substitutes for the leaf extract. The study of the active principle in leaf extracts was not pursued further. It is thermolabile and not identifiable as a ferric salt.

Further study (78, 80) of the simultaneous evolution of oxygen and reduction of ferric oxalate revealed certain characteristics in common with photosynthesis, e.g., similar effects of light intensity and flashing light and inhibition by urethane. An experimental limitation was that the reduced ferrous oxalate would take up (oxygen pressure above ~4 mm. mercury) the oxygen produced in the light. Later

work (81) showed that if ferricyanide were provided to reoxidize the ferrous oxalate, oxygen could be measured manometrically. French & Anson (79) have extended the manometric study of this phenomenon, which they appropriately call the Hill reaction. In their hands the large rate of oxygen evolution may continue for an hour or more but finally comes to a halt. The inactivation is greatly accelerated by heat; its cause is as yet unknown. Because of the high temperature of inactivation and the inhibitory effects of specific enzyme poisons and protein denaturants, they believe that a protein and probably an enzyme are involved. The Hill reaction clearly calls for further study.

Theories

There have been numerous theories of limited scope directed toward the explanation of the results of one or another type of experiment [e.g. (53, 58, 73, 80)]. In addition, there have been several attempts to fashion a picture of the photosynthetic mechanism so extensive as to encompass all, or at least a large number, of the existing data. All such older theories and some of the later ones are conditioned by the limitation of a four-quantum process. It now seems that such a limitation is not required. There are a number of peculiarities of photosynthesis to be explained; for instance, (*a*) highest efficiency at lower light intensities, (*b*) a short induction period becoming unmeasurably short at low intensities, (*c*) discrepancy between the speeds of the light (or dark) reaction as calculated from flashing-light experiments on the one hand, and the light saturation curves on the other.

Two general types of theories have been developed to explain these salient features. One depends upon a photosynthetic unit and postulates a limited number of centers [number $\backsim(1/2000) \times$ chlorophyll molecules] at which chemical reaction takes place. Wohl (66, 82) supposes the required co-ordination between chlorophyll molecules to be achieved by an optical-physical unit. Ornstein *et al.* (83) and Wassink & Katz (59) picture a different type of co-ordination accomplished by acceptors, present always in abundance, which carry the energy of activation from the many chlorophyll molecules to a limited number of reducing centers. A common feature of this general type of theory is that intermediates are thought to be present in concentrations several orders of magnitude less than that of chlorophyll.

The other type of theory holds that concentration of intermediates and of chlorophyll is of the same order of magnitude and that energy

of activation of individual chlorophyll molecules is transferred indirectly [Briggs (49)] or directly [Franck & Herzfeld (84)] to an acceptor-intermediate complex. The remarkably ingenious and comprehensive theory of Franck & Herzfeld [(84), see also (3)] has been applied to the interpretation of fluorescence and induction phenomena (52), flashing-light data (46), and observations on photooxidation (12). They believe that a direct energy exchange between chlorophyll molecules and carbon dioxide or intermediates must take place since sudden changes in carbon dioxide concentration produce such rapid fluorescence effects.[6] They describe the dark reactions in terms of three catalysts controlling the formation of the carbon dioxide acceptor or intermediate-acceptor complex ("catalyst A"), the stabilization of the photoproducts of the light reaction ("catalyst B"), and the production of free oxygen ("catalyst C"). The short induction period is explained by the occurrence of back reactions which keep the population of the various intermediates at an approximately constant level even in the dark.

The generalized concept of van Niel (63), based on the comparative biochemistry of carbon dioxide reduction, has had additional experimental corroboration (64, 76). Certain features of this concept are incorporated in the theory of Franck & Herzfeld.

In the arbitrary photochemical theory proposed by Baly (85) recent experimental data have been studiously ignored.

[6] Note, however, that a similar argument has been used by Kautsky & Eberlein (58), though on less adequate data, for the participation of an oxygen complex in the energy transfer.

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MINERAL NUTRITION OF PLANTS

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The continuous consideration which the Editors of the *Annual Review of Biochemistry* have given to the subject of Mineral Nutrition of Plants imposes upon the present reviewer chiefly the responsibility for considering the contributions to this subject which have reached his library during 1942. It is deemed wise to follow the precedent set by previous reviews and no attempt will be made to compress within the limits of assigned space all the papers in this rather broad field of inquiry which appeared during the past year. It is proposed to discuss a series of topics which can be broadly grouped under two main headings: (*a*) absorption and accumulation of ions and (*b*) interrelations and functional aspects of nutrient elements. Special mention will also be made of diagnostic methods for assessing nutrient deficiencies and of the present status of micronutrient elements.

Absorption and Accumulation of Ions

Previous reviews have consistently kept pace with the progress in this subject which continues to occupy a central position in the field of inorganic nutrition of higher plants. In the past year, the previously formulated basic concepts of salt absorption by most cells against a concentration gradient have been further tested by Hoagland & Broyer (1) with the use of different experimental techniques. In their former studies, these investigators have followed the process of absorption and accumulation of ions by analyzing the expressed sap of root tissues after killing them by rapid freezing at the end of the experimental period. In the present investigation they undertook to compare the composition of the expressed sap from killed root tissues with that of the exudate from living and actively absorbing, intact roots. The latter was obtained by decapitating the shoots of barley and tomato plants a short distance above the junction of root and stem

and collecting the bleeding sap either directly or by applying suction to the cut end. There are reasons for believing that this exudate is derived primarily from the xylem vessels whereas the expressed sap from frozen roots, although it has proved useful and fruitful of results, must necessarily be regarded as a composite fluid of many tissues.

It is significant that the independent method of exudate analysis has led to the same conclusions as the earlier studies concerning the nature of salt accumulation by higher plants. The following facts were demonstrated by analyzing the exudation fluid: (*a*) Physiologically active roots are able to absorb and accumulate mobile ions such as potassium and bromide against a concentration gradient. The concentration of mobile ions in the exudate may be of the same order of magnitude as in the expressed sap (after freezing) of the roots from which the exudate was derived. (*b*) The salt-accumulating power of the roots is a function of their aerobic metabolism and is lost under conditions of oxygen deficiency. A number of experiments were carried out to elucidate the relation between aerobic respiration and salt accumulation. Hoagland & Broyer found no support for the view that respiration is effective through the production of carbon dioxide and carbonic acid, which has figured in certain proposed mechanisms for ion absorption. Certain treatments, such as the addition of methylene blue or cyanide to the substrate, inhibited accumulation without greatly depressing carbon dioxide production. The suggestion is made by the authors that although the exact nature of the system is not yet revealed, the process of salt accumulation is linked with a metal-catalyzed aerobic respiratory system. Indirect evidence also renders unlikely the possibility that failure of salt accumulation in an anaerobic environment is due to the injurious effects of such by-products of fermentative processes as lactate and ethyl alcohol; however, more experimental data are wanted.

A further confirmation of the importance of aerobic metabolism in salt accumulation is afforded by an experiment in which the usual salt gradient between root and substrate was reversed, i.e., the initial salt concentration in the nutrient medium was higher than the initial salt concentration in the root. But even when the inward gradient for potassium and bromide was thus rendered positive, very little of either was absorbed by the root anaerobically, and in no case was the external concentration approached within the plant. On the other hand, a rapid salt absorption and even accumulation to a higher con-

centration than that of the relatively concentrated external solution was obtained under aerobic conditions.

These conclusions are in harmony with another line of evidence obtained by Hoagland & Broyer (1) from a series of experiments with the unicellular alga, *Nitella,* in which, following earlier studies by Brooks, radioactive isotopes of rubidium and bromide ions were used. A rough separation of vacuolar sap from protoplasm and cell wall was made and ionic absorption was determined in each. Despite certain experimental difficulties their data can be interpreted as indicating a rapid ionic accumulation in excess of the external concentration in the protoplasm, followed by a continued movement into the vacuole until the concentration there exceeds that in the protoplasm. As with the root tissues of higher plants, ionic accumulation in the vacuole of *Nitella* did not take place in an anaerobic environment.

On the basis of these results Hoagland & Broyer suggest that a pump-like, secretory action on the part of the protoplasm is responsible for the transfer of increasing amounts of salt into the vacuoles of cells which possess the capacity for accumulation. This secretory activity of the protoplasm would obviously depend on the maintenance of favorable conditions for aerobic metabolism. Thus, the concept which links the metabolic activity of cells with accumulation of ions envisages two stages in the process of accumulation in which energy is expended: first, the absorption by the living protoplasm;[1] and second, the transfer of the absorbed ions into the vacuole. As far as the absorbing roots of higher plants are concerned, the secretory activity of the protoplasm is thought of as introducing the absorbed ions from the region of living cells in the cortex, across the endodermis into the xylem.

It is axiomatic that attempts to formulate generally valid theories of absorption and accumulation of ions by plants must rest on a solid foundation of experimental facts. The tendency has been at times, however, to offer generalizations which lay claim to wide applicability but which have been postulated on the basis of results obtained with only a few or even one type of plant cell or tissue system. The inherent complexity of the problem of absorption and the extent to which certain conclusions resting on the behaviour of one species may not apply to another is illustrated by the important work of Collander

[1] This stage embraces permeability with its dependence on the metabolic activity of the cell.

(2). Collander investigated the selective cation absorption of some twenty higher plants of varying ecological types. These were grown for about two months in complete nutrient solutions containing several cations in equivalent amounts, and the plants were then analyzed spectrographically for the metallic elements in question. The absorption of sodium, potassium, rubidium, magnesium, calcium, strontium, and manganese was studied most closely, that of lithium and cesium less extensively. It was found that while a given species exhibited for the most part a consistent type of selective absorption from among the several ions, wide differences in relative selectivity were encountered among the various species. Thus while all halophytes, but one, were distinguished by their accumulation of sodium, plants like buckwheat, corn, and sunflower were noted for their pronounced exclusion of this element. Again all *Chenopodiaceae* as well as buckwheat were remarkably rich in magnesium while calcium and strontium were absorbed in the greatest amounts by sunflower, buckwheat, and white mustard; oats, corn, and spinach absorbed calcium and strontium in least amounts. These absorptive characteristics of various species were essentially unchanged by environmental variations, and similar relations were obtained when plants were grown in soil rather than in nutrient solution.

The variations in the absorption characteristics of the different species were so great that it is impossible to arrange the absorption of cations in an order that would be valid for all the plants investigated. For example, the maximum values for sodium absorption were twenty to sixty times greater than the minimum; the maximum values for lithium, magnesium, calcium, or strontium were three to five times greater than the minimum. If generalizations are sought, however, the following may be listed as applying to most plants: potassium, rubidium, and cesium are the ions most readily accumulated, followed usually either by strontium and calcium or, about as frequently, by magnesium, lithium, sodium, and manganese. As far as the effect of one ion on the absorption of another is concerned, Collander found that two pairs of ions had a mutually depressing effect: potassium and rubidium were one, and calcium and strontium the other. Interestingly enough, the ratio of absorption of the two ions in each pair remained constant over a wide range of concentrations. It was one when the two ions in each pair were present in equal concentration in the outside solution, and it varied in the same proportion in which the relative concentrations were altered in the external solution. Col-

lander suggests that each pair of ions may be regarded as in effect representing physiological "isotopes," i.e., the plant is unable to distinguish between potassium and rubidium or between calcium and strontium and absorbs them in the same ratio in which they present themselves at the absorbing surfaces.

The selective absorption of ions has provided the basis for advancing various hypotheses as to the nature of the semipermeable protoplasmic membrane, which hypotheses have sometimes rested on the assumption that bivalent ions like calcium, magnesium, and strontium are absorbed less readily than monovalent ions like sodium. It is interesting to note that Collander found the reverse to be true for most of the plants he investigated.

The bivalent ions, particularly calcium, were found by Viets (3) to increase appreciably the absorption of the univalent ions, potassium and bromide, by excised barley roots. Calcium increased the absorption of potassium by 80 and that of bromide by 100 per cent without affecting the rate of carbon dioxide production. Calcium was always more effective than magnesium, and magnesium more effective than strontium in solutions of like concentration. Mixtures of calcium and magnesium sulfates produced increases of the same general magnitude as did these salts when applied singly, which suggests that these bivalent ions increased the permeability of the plasma membrane to potassium and promide absorption.

In another paper Collander (4) reported the distribution of cations between the root and shoot of about sixteen plant species grown in water culture. He found that on the whole the shoot contained about ten times as much total cations as the root while the relative proportion of the cations varied as follows:[2] potassium, rubidium, cesium, and magnesium were found in about the same equivalent percentages in the roots as in the shoots; sodium and manganese were found in most species in higher equivalent percentages in the roots than in the shoots, whereas the reverse was true for calcium,[3] strontium, and lithium.

The problem of the distribution and transfer into the shoot of ions

[2] Relative proportion was expressed as the "equivalent percentage" of a cation, i.e., its percentage of the total cation equivalents in a kilogram of dry tissue.

[3] Some doubt may arise whether calcium precipitated on the surface of the roots may not be included in the total root calcium.

absorbed by the root is considered in a paper by Steward, Prevot & Harrison (5). These authors found that the apical regions of barley roots, which have the greatest capacity for accumulation of ions from an external solution, are also the regions from which the transfer of the absorbed ions into the shoot proceeds most readily. In harmony with their earlier results on bromide accumulation, they found also that the accumulation of cations, potassium and rubidium, proceeds in a longitudinal gradation along the root, with the greatest accumulation taking place at the apex. This was found to be the case whether the roots were attached or detached from the shoots. A difference was noted in the relative distribution of rubidium and bromide in the shoot and root. From an aerated rubidium bromide solution, intact barley plants were found to accumulate in their roots a higher concentration of rubidium than of bromide. Bromide, however, moved more readily into the shoot than rubidium.

The unequal absorption of cations and anions by roots as linked to regulatory changes in metabolism was investigated by Ulrich (6). The total nonvolatile organic acid content of excised barley roots containing an ample supply of available sugar remained constant unless a change in the ionic balance took place within the roots. When the root absorbed an excess of cations over anions, organic acids were formed, and, conversely, when the absorption of anions exceeded that of cations, organic acids disappeared. The respiratory quotients reflected these organic acid changes by increasing as the acids disappeared and by decreasing as they were formed. It occurs to the reviewer that some interest may be attached in exploring the possible connection betweeen the increase in the respiratory quotient, found by Ulrich to be associated with excess anion absorption and with the resultant organic acid disappearance, and the "anion respiration" of Lundegårdh & Burström discussed in an earlier review [cf. (7)].

Further support for the view that the organic acid metabolism in the roots is principally concerned in the maintenance of the acid-base balance was found by Ulrich (6) in the failure of temperature and variations in oxygen tension to affect the total organic acid content of the roots, so long as these environmental factors did not influence the ionic balance within the absorbing cells. As for the formation of organic acids in the plant, the evidence seemed to favor the conclusion that they were derived from the carbohydrates rather than from the oxidative deamination of amino acids.

Mechanism of ionic absorption from the nutrient medium.—The

preponderance of evidence at the present time leaves no doubt that the process of salt accumulation is intimately associated with the metabolic activities of the cell and that it cannot be regarded as one merely involving a passive entry of ions into the roots. There remains, however, the interesting, and from the standpoint of agriculture, important question, by what mechanism the transfer of nutrient ions from the soil particles into the plant root is accomplished. The metabolic aspects of salt accumulation have been investigated almost exclusively with the aid of the nutrient solution technique. This experimental approach has rested on the assumption, until recently unchallenged, that a basic similarity exists between the absorption of ions from a nutrient solution and the absorption of nutrients from a soil solution. It was envisaged in both instances that the ions in the solution surrounding the roots are free to move unhampered by surface forces of colloidal particles. The solid phase of the soil was regarded as the ultimate source of most ions, particularly cations, in the soil solution. In recent years, however (see previous reviews), important evidence has been put forth for the "contact exchange of ions" as an alternative view of ion absorption by roots. This theory, as formulated by Jenny & Overstreet, considers the absorption of ions as a process involving the transfer of ions from the surfaces of the solid colloidal particles of the soil to the surfaces of the root, essentially without the intermediary of the intermicellar liquid or solution phase.

Several contributions in the past year dealt with the mechanism of ion absorption by roots. Schlenker (8), extending his earlier work with adsorbed ions, showed that the principal nutrient ions, when added in an adsorbed form to a quartz sand substrate, can support the growth of young kidney beans, corn, and buckwheat. Potassium, calcium, and magnesium were adsorbed on permutite, and nitrate, sulfate, and phosphate on aniline black. A comparison of equal amounts of soluble salts and adsorbed ions indicated that the adsorbed ions produced more growth. It would seem necessary, however, to explore a wide range of concentrations of the individual ions before the greater efficiency of one source over another can be regarded as established. Thus, Overstreet *et al.* (9) found that with clay suspensions containing 1 to 5 m.eq. per l. of adsorbed potassium the rate of potassium uptake by the plant was only 25 to 50 per cent of that from comparable salt solutions.

Albrecht, Graham & Shepard (10), from a series of computations, have concluded that a crop absorbs more calcium from a soil than is

present on the surfaces of the clay particles which are in immediate contact with the absorbing roots. They have suggested that the needs of the plant are met by a process in which the clay particles in contact with the roots in turn replenish the calcium they lose to the plant by means of another ionic exchange with the minerals of silt size and with other clay particles not in contact with the roots. Albrecht *et al.* made their calculations on the assumption that the cations absorbed by the plant are derived solely through ionic exchanges between the roots and the adhering clay particles. As will be discussed more fully later, the presence of moisture in soil, indispensable for crop growth, brings about ionic exchanges between the cations (including hydrogen ion) present in the soil solution and the clay particles, which are not necessarily in contact with the roots. As the plant absorbs water and nutrients from the soil solution, it draws, in effect, on cation reserves from colloidal particles not immediately adjacent to the root surfaces. From this point of view the supply of cations to the plant does not seem to depend directly on cation exchange among the various solid soil particles, as postulated by the authors (10).

Overstreet, Broyer, Isaacs & Delwiche (9) compared the absorption of potassium by excised barley roots from nutrient solutions and from colloidal suspensions of potassium-bentonite. They found that barley roots absorbed potassium from a potassium-bentonite suspension much more readily than was possible if absorption took place only from the intermicellar liquid or the liquid phase of the suspension. Their data indicate that the potassium adsorbed on the clay particles was included in the total potassium taken up by the root. Overstreet *et al.* consider two possible mechanisms by which the adsorbed potassium was made available to the roots, one not requiring and the other requiring the intervention of a liquid phase. The first mechanism, the contact exchange mechanism, states that given a proximity of surfaces between roots and colloidal particles, the "ionic swarms" of the two would become intermingled and a net intake of potassium by the roots would thus be directly effected. The second mechanism pictures absorption as proceeding from the liquid phase or the intermicellar fluid of the suspension, with carbonic acid acting as a carrier of potassium ions between the clay and root surfaces by means of the following two steps:

$$\begin{aligned} &(a)\ \boxed{\text{clay}}\ \text{K} + \text{H}_2\text{CO}_3 \rightarrow \boxed{\text{clay}}\ \text{H} + \text{K}^+ + \text{HCO}_3^- \\ &(b)\ \text{H} - \text{root} + \text{K}^+ + \text{HCO}_3^- \rightarrow \text{K} - \text{root} + \text{H}_2\text{CO}_3 \end{aligned} \qquad \text{(I)}$$

Overstreet *et al.* point out that while cation exchange is consistent with present concepts of colloid chemistry, the carbonic acid mechanism falls short of explaining the results of certain experiments on sodium absorption. Step (*b*) in the carbonic acid mechanism demands that cation absorption from a bicarbonate solution should be equal to its absorption from a carbon dioxide-saturated cation-clay suspension of the same concentration. This was not found to be the case for sodium in an earlier experiment by Overstreet & Jenny (11) in which they found, particularly for low concentration ranges, a greater absorption from a sodium-clay than from a sodium-bicarbonate solution of similar sodium concentration. It does not appear, however, that the results with sodium, an ion meagerly absorbed by most plants (2), are representative of the absorption of other cations from a soil solution. In the present paper (9), for instance, Overstreet *et al.* report a reverse situation for potassium: its absorption from a clay suspension was only 25 to 50 per cent of that from a comparable salt solution. Moreover, under soil conditions the influence of nitrate ion in promoting cation absorption needs to be considered.

What seems essential for the functioning of intermicellar liquid in soils as a carrier of cations to the roots is a continuous supply of hydrogen ions to displace the cations from the clay particles. An anion other than bicarbonate can participate in step (*b*) of equation I. The supply of hydrogen ions is brought about by the metabolic activity of the roots, of which the evolution of carbonic acid is but one phase. Overstreet *et al.* (9) cite data to show that organic acids synthesized by the plant, rather than carbonic acid, are the ultimate sources of the hydrogen ion which replaces the adsorbed cation on the clay. The conclusions of Ulrich (6) about the regulatory function of organic acids when ions of opposite charge are absorbed in unequal proportions and the findings of Hoagland & Broyer (1) about the lack of a necessary correlation between carbon dioxide production and salt absorption will be recalled in this connection.

The paper of Overstreet *et al.* provides an appropriate point of departure for the reviewer to attempt an appraisal of the present status of the nutrient solution and the contact exchange mechanism of ionic absorption by roots. It is essential to recognize at the outset that while both theories of ionic absorption are based on experimental data procured with simplified systems under controlled laboratory conditions, they represent important experimental approaches to the study of nutrient availability and absorption from soils. The following may be

listed as some of the salient facts bearing on the applicability of the two theories to nutrient absorption by roots from soils:

A. Higher plants have been grown under laboratory conditions with the inorganic nutrients supplied either as adsorbed complexes or in a nutrient solution. As far as the nutrient solution technique is concerned, overwhelming evidence from a host of workers over more than three quarters of a century leaves no doubt that not only can a large number of diverse plant species complete their life cycle in this medium but also that excellent crops equaling or even exceeding those obtained from fertile soils can be produced. [Compare Arnon & Hoagland (12).] The very recent history of "contact feeding" cannot obviously muster an equally impressive array of evidence. But the work of Schlenker (8), already cited, indicates that all the principal nutrient ions can be supplied with good results to higher plants in an adsorbed form, and there seems to be no reason at present to question that in due time many plants will be grown from seed to seed by this method.

B. The naturally occurring colloids in most soils are negatively charged and contain the nutrient cations in an adsorbed form. On the other hand, the anions, chiefly nitrate and sulfate, occur in soils as free ions in the soil solution.

C. The presence of the anions, nitrates, sulfates, and often of important amounts of chlorides in the soil solution demands the simultaneous presence of equivalent amounts of ions of opposite charge, which include the important nutrient cations. To use the symbols represented in equation I, the following sample reaction takes place between the cations adsorbed on the clay particles and the soil solution as nitric and sulfuric acids are produced in the soil as a result of microbiological activity:

$$\boxed{\text{clay}}\,K + H^+ + NO_3^- \rightarrow \boxed{\text{clay}}\,H^+ + K^+ + NO_3^- \qquad \text{(II)}$$

The quantitatively very great amounts of nitrate used by the plant during its growth period insure the presence of important amounts of cations in the soil solution.

D. Available soil moisture is a prerequisite for plant growth in soil, or stated in other terms, the continuous presence of soil solution is indispensable for normal plant growth, quite apart from its nutrient composition.

It seems clear that while it has been shown experimentally that

plants can absorb nutrients either from a solution or from a colloidal adsorption complex, the question of greatest interest to chemists and physiologists is which mechanism is the dominant one under natural conditions of plant growth in soil. Because of the considerations outlined under B, C, and D, it is the reviewer's view that the new evidence on cation exchange does not detract from the important position heretofore accorded to the soil solution in discussions of soil fertility problems.

It is reasonable to expect, however, that this valuable new point of view on contact absorption will lead to explanations of some soil-plant phenomena which are at present obscure. To quote from a paper by Hoagland & Arnon (13),

> Frequently, ... , soil solution concentrations of potassium and phosphate are very low at any moment and rapid entry of these ions into the soil solution from solid phases would often be required to explain on a soil solution basis their actual absorption by the plant. But again we are not yet sure that in soils capable of supplying enough potassium to a crop, the low concentration of potassium sometimes occurring in the soil solution is necessarily inadequate. We recall the capacity of active roots for rapid absorption of potassium from extremely dilute solutions and the potentialities for root surface development.

The complexity of the problem of nutrient absorption by plants is by no means exhausted by a discussion of the solution and the contact exchange theories. Questions of nutrient availability in soils arise which are not covered by either theory. For example, it has been shown [cf. (13)] that in certain soils plants can absorb potassium from the nonreplaceable potassium fraction, in the sense that amounts of this element equivalent to those absorbed by the plants were not recovered in leachings of the soil with ammonium acetate or dilute acids. According to present views it is difficult to explain the absorption of nonreplaceable potassium, which sometimes occurs, either through contact exchange or absorption through the soil solution.

A somewhat different approach to the mechanism of nutrient absorption by roots from soils is found in a recent paper by Lundegårdh (14) in which he summarizes the results of his earlier investigations of the electrical charges of grass roots. Lundegårdh develops a theoretical concept for which experimental illustrations are cited, envisaging the surface layer of the protoplasm in the absorbing roots (Z-layer) as an amphoteric colloid with predominantly acidic properties, due to the hydrogen ion of metabolic origin. (Mathematical formu-

lations are given for the theory of the electrochemical properties of the surface of living roots.) The Z-layer is separated from the soil colloids by a neutral layer of cell wall. The hydrogen ions on the Z-layer pass through the cell wall and exchange for the cations derived from the soil colloids. Likewise, the nutrient anions, nitrate, dihydrogen phosphate, etc., are thought to displace hydroxyl or bicarbonate ions from "the points of negative valency in the Z-layer."

Thus far Lundegårdh's picture of ionic absorption fits in with some of the postulates of the contact exchange hypothesis. But Lundegårdh regards the actual transfer of ions as being accomplished through the soil solution. To quote him directly:

> Nutrient ions . . . . must be detached from the surface of the soil colloids and pass through the aqueous cell walls before they can be absorbed by the living protoplasm. The soil particles seldom form a continuous layer, coating the outside of the root hairs, but are irregular with intervening spaces covered by an *aqueous layer* (Lundegårdh's emphasis) or, after watering, by a film of soil water. The aqueous film between the soil colloids and the absorbing surface of the roots is, therefore, always a factor in the process of the uptake of mineral nutrients by the plant.

Effects of hydrogen ion concentration on plant growth.—Albrecht & Schroeder (15) have investigated the effect of hydrogen ion concentration on the absorption of cations, particularly calcium, by potato and spinach from a soil, the colloidal fraction of which was treated to provide various proportions of cations. Two hydrogen ion concentrations were investigated, one corresponding to a soil pH of 5.2 and the other to a pH of 6.8. The absorption of cations at the two pH levels, from a given cation saturation percentage of the clay fraction of the soil, was studied by analyzing the plant at the end of the growth period. The analyses disclosed that calcium, magnesium, strontium, and manganese were absorbed in larger amounts from the soil at pH 5.2 than at pH 6.2. In this paper the authors conclude that hydrogen ion in the soil facilitates the absorption of cations. In another paper, Schroeder & Albrecht (16) call attention to the relatively high calcium content of potato shoots as compared with the high potassium content of potato tubers. They suggest that the generally known reduction of the incidence of potato scab (a fungus disease affecting potato tubers) in acid soils is due to a greater intake of calcium and potassium by the plants at acid reactions, in accordance with the conclusions of their previous study (15).

It should be pointed out that an acid reaction is in many soils an indication of a deficiency of calcium brought about by leaching and the resultant replacement of calcium from the colloidal particles by hydrogen ions. In all such cases of calcium deficiency, this element must be added to the soil to permit plant growth regardless of the resultant shift toward alkalinity in the soil. It is conceivable that under such experimental conditions as described by Albrecht & Schroeder (15), where an adequate calcium supply was accompanied by a distinctly acid reaction in the soil (pH 5.2), a higher concentration of calcium was possible in the soil solution than at pH 6.8 (through the exchange of hydrogen ion of the solution with the calcium ion on the clay). A higher concentration of calcium and other cations in the soil solution would in turn account for the increased absorption at acid reactions as reported by Albrecht & Schroeder (15).

Arnon & Johnson (17) undertook the investigation of the influence of hydrogen ion concentration on the growth of higher plants by means of a technique which permitted identification of the effects of external pH in terms of an isolated variable. A range of pH from 3 to 9 was selected. In order to distinguish between the direct effects of hydrogen ion concentration on the plant and its indirect effects through the alteration of the composition of the nutrient medium, they grew their plants by means of a modified nutrient solution technique which included the following features: constancy of composition of the nutrient solution with respect to the principal nutrients throughout the range of pH 3 to 9; the supply of iron and manganese as humate-metals to insure their availability at alkaline reactions; and maintenance of the initial pH of the external medium generally within $\pm$ 0.2 pH units.

For such unrelated species as lettuce, tomato, and Bermuda grass, profoundly adverse effects of hydrogen or hydroxyl ion concentration were found only at extremes of acidity or alkalinity. As far as the sap of either shoots or roots is concerned, Arnon & Johnson (17) found no significant effect of the external reaction on the pH of expressed sap, with the possible exception of extremes unfavorable for growth. With all three plants complete failure of growth occurred at pH 3 and a marked decline was observed at pH 9. But within a relatively wide range of pH, between 4 and 8, fluctuations in the hydrogen ion concentration were tolerated provided an adequate supply of all the nutrient ions was maintained. A similar conclusion may be reached from the results of Edgerton (18) who has grown apple seedlings in

buffered nutrient solutions ranging in pH from 4.0 to 7.5. At the higher pH values iron chlorosis and markedly decreased growth were in evidence unless sodium metaphosphate was added to the nutrient solutions. The addition of sodium metaphosphate prevented the precipitation of iron at the higher pH values and made possible normal growth and freedom from iron deficiency symptoms. The unfavorable growth initially observed at the higher pH values was thus shown to be due not to the hydroxyl ion concentration but to the insufficiency of iron.

In a companion study, Arnon, Fratzke & Johnson (19) set out to investigate the effect of external pH on the absorption of calcium, magnesium, potassium, nitrate, and phosphate ions. The object was to determine whether the observed effects of pH on growth, particularly at pH 3 and 9, are explicable by the influence of hydrogen ion on nutrient absorption. The general plan of the experiments provided for the use over short periods of time of relatively uniform healthy plants with well-developed roots, which were grown for five weeks prior to the absorption tests in the same complete nutrient solution of a pH favorable to growth. For the absorption experiment the plants were transferred for ninety-six hours into a series of nutrient solutions ranging in gradations of one pH unit, from pH 3 to 9.

In accord with the previously discussed pH effects on growth, injury to roots was observed at pH 3 within less than an hour. At this reaction, no absorption, in fact suggestions of loss, of calcium and phosphate was in evidence. For tomato and lettuce this reaction was also wholly unsuited for the absorption of other ions. Evidence was also obtained of lower calcium absorption, particularly by tomato and lettuce, from strongly acid solutions (pH 4 and 5) than at higher pH values. The absorption of magnesium, potassium, and nitrate was little affected by the external reaction between pH 4 and 9. On the other extreme of the pH scale, at pH 9 no untoward external effects were found, nor was there at pH 9 interference with nutrient absorption with the important exception of phosphate, the absorption of which was markedly decreased. It is suggested that the deleterious effects of hydrogen ion concentration on the absorption of calcium and phosphate at pH 3 and phosphate at pH 9 can account, regardless of the mechanism by which these effects are brought about, for the poor growth at these reactions. It is not implied, however, that interference with absorption is necessarily the sole factor involved in bringing about injury at extremely acid or alkaline reactions.

Interrelations and Functional Aspects of Nutrient Elements

Drift in plant composition.—Phillis & Mason (20) have presented evidence on the diurnal variations in the mineral content of cotton leaves. All the mineral elements with the exception of calcium were found to increase in the leaves during the day and decrease at night. Calcium, while it increased during the day as the other elements, did not show a significant change at night. The results were interpreted as supporting the view that the mineral elements enter the leaf with the transpiration stream in the xylem and, excepting calcium, are exported at night in the phloem. An interesting observation was reported on the important loss of potassium in night exudation which was found to be chiefly a solution of potassium bicarbonate.

Mason & Phillis (21) have also investigated certain relations between the supply of mineral nutrients and the succulence or hydration of leaves. They found that the conflicting reports in the literature about the effect of potassium on foliar hydration (see previous reviews) can be reconciled if the effects of various levels of supply of this nutrient on the size of the plant and the accompanying moisture supply are considered. When an increase in size of the plant as a result of an increased potassium supply occurred under conditions leading to a water stress, there was a decrease in the degree of hydration of the tissues. When, however, the increase in size was not accompanied by a water stress, a positive correlation was found between a higher potassium supply and hydration.

In another paper, Mason & Phillis (22) have reported a very interesting correlation between the swelling of discs punched from cotton leaves and the salt content of the solutions in which they were floated. They found that the discs floating in daylight on salt solutions may show very large increases in water content (about 170 per cent in thirteen days) but that discs floating on water showed a loss of water in the same period of time (about 20 per cent). The positive effect of salt on water uptake occurred with either a full nutrient solution or calcium chloride solutions. Although the swelling of leaf discs in concentrated salt solutions has not yet been investigated, the authors found that with the concentrations used, swelling was proportional to the amount of salt absorbed.

Further investigations are in progress, but on the basis of present observations Mason & Phillis suggest that the hydration capacity of

leaf proteins is increased by salts in the same way as the hydration capacity of gelatine in the isoelectric region. Also, the hydration capacity of protoplasm may be influenced by changes in respiration associated with salt absorption. It should be noted that while swelling was observed with other dicotyledons, it could not be demonstrated in monocotyledons.

Rohrbaugh (23) examined with the aid of fluorescent lamps the influence of light quality on the distribution of certain mineral elements in the bean plant. The calcium content of the leaves, which was inversely related to that of the stems, was highest in plants having the highest dry weight, i.e., those receiving their illumination from most of the visible spectrum. It decreased when narrower regions of the spectrum were used. Phosphorus content appeared to decline with the inclusion of either the blue or the red end of the spectrum. It was highest when light from both ends of the spectrum was omitted. Potassium and magnesium content showed no significant change with variations in the quality of light.

Aspects of nitrogen metabolism.—One of the interesting questions of nitrogen metabolism in higher plants is the ability of detached leaves to synthesize proteins. There is support in the literature for the view that isolated leaves, particularly mature ones, lose the power of protein synthesis upon removal from the plant. This would suggest that the factors governing the synthesis of protein in a leaf are not solely determined by the enzymatic systems and the nonprotein nitrogen fractions in the leaf, but also by some extraneous agent, presumably of a hormonal nature. The problem was investigated by Phillis & Mason (24). They compared the protein synthesis of intact cotton leaves with that of discs punched from leaves and floated on a nutrient solution containing nitrate nitrogen. They found that the detached leaf segments were able for several days to synthesize protein as readily as the intact leaves. Protein synthesis was found to be dependent on the level of the nonprotein (crystalloid) nitrogen rather than on the total nitrogen in the leaf. As the level of crystalloid nitrogen (crystalloid nitrogen per 100 gm. dry weight) increased, the level of protein nitrogen (protein nitrogen per 100 gm. dry weight) increased to a maximum and then declined with a further rise in the crystalloid nitrogen. Phillis & Mason point out that this relation between the crystalloid nitrogen and protein nitrogen is reminiscent of a type of apolar adsorption in which the amount of adsorbed material rises at first with increasing concentration, reaches a maximum, and then de-

clines with still higher concentration. They suggest that the crystalloid nitrogen is similarly adsorbed before it is converted into protein and that "adsorption plays a dominant role in determining the equilibrium between protein and crystalloid-N."

As Phillis & Mason remark, "it is at least surprising, in view of the heterogeneity of the crystalloid fractions, that this relation between protein and crystalloid-N should hold over such a wide range." It would be of interest, therefore, to have a breakdown of the crystalloid nitrogen at least into organic and nitrate nitrogen fractions in order to establish with greater precision the reported correlation with protein synthesis. Also, the carbohydrate status of the detached leaves would seem particularly relevant.

Phillis & Mason (24) report, on the basis of more limited data, evidence of similar relations between polysaccharides and total sugars as well as between insoluble and soluble phosphorus. They suggest that these equilibria are likewise governed by adsorption. It should be noted that all their data were obtained on leaves from young plants still in the vegetative stage or just beginning to flower.

Stanley's identification of tobacco-mosaic virus with a crystalline globulin linked the subject of virus activity with the nitrogen metabolism of infected plants. Spencer (25) investigated the influence of nitrogen supply on the rate of multiplication of tobacco-mosaic virus. He found that by the eighth day after inoculation the juice from the high-nitrogen plants contained twelve times more virus than that from the low-nitrogen plants, even though the high-nitrogen plants were only two or three times as large. The increased virus activity associated with an increased nitrogen supply is ascribed primarily to an increase in the rate of virus multiplication and only slightly, if at all, to virus inactivation in the low-nitrogen plants.

In another paper (26) Spencer reported on the effect of age of lesion and of the nitrogen supply in the nutrient medium on the specific biologic activity of the tobacco-mosaic virus. In young lesions the virus displays only a fraction of its potential biological activity. The virus increases in the inoculated leaf both in amount and activity for as long as twenty days after inoculation. Nitrogen starvation inhibits further virus increase. The inhibition of the specific activity takes place, however, before the further increase in virus protein itself is brought to an end.

Stanley (27) has obtained, by growing virus-infected plants in a nutrient medium supplied with radioactive phosphorus, a tobacco-

mosaic virus protein containing radioactive phosphorus. Since the radioactive phosphorus could not be separated from the virus protein by ultracentrifugation, it was evidently bound in the protein molecule, presumably in the nucleic acid fraction. Stanley then inoculated the "radioactive virus" into healthy plants in an attempt to learn by tracing the radioactive phosphorus the thus far uncertain mode of virus reproduction: whether by fission or by some sort of an autocatalytic process. Unfortunately, the results of this interesting approach to the fundamental problem of virus reproduction proved inconclusive. Following inoculation into healthy plants, most of the radioactive phosphorus originally present in the virus protein was found to be associated with nonvirus components. Of the radioactive phosphorus in the nonvirus components, 40 per cent was found in the inoculated and 60 per cent in the uninoculated portions of the plant. Although a small but significant amount of activity was found in the virus isolated from the uninoculated portions of the plant, it was impossible to conclude that it was derived from the virus originally injected since it might have been due to radioactive phosphorus which was transported in nonvirus form and later incorporated into the virus protein.

The utilization of ammonium versus nitrate nitrogen by plants continues to receive attention. Talley & Blank (28) found that the root rot fungus, *Phymatotrichum omnivorum,* was able, similar to higher plants, to utilize ammonium nitrogen if proper safeguards were used to prevent injury from the physiological acidity of the ammonium salts. There was no evidence of ammonium toxicity *per se*. This fungus was also able to grow well with nitrite nitrogen. Walp (29) found that the alga, *Nostoc muscorum,* was able to assimilate either ammonium or nitrate nitrogen, but nitrate nitrogen affected cell proliferation more than ammonium nitrogen.

Chapman & Parker (30) kept for over three years a record of weekly absorption of nitrate by two young Valencia orange trees grown outdoors in a nutrient solution. Absorption was related to the temperature of the air and of the nutrient medium. Nitrate absorption was at a minimum during January and February and reached its highest values during late spring, summer, and early fall.

Reciprocal relations of elements.—The long discussed question whether sodium can replace potassium in the plant was considered by Mullison & Mullison (31). These investigators found evidence of partial substitution of potassium by sodium in the delay in the onset of potassium deficiency symptoms when sodium was added to potas-

sium-deficient sand cultures. It remains to be shown what particular function of potassium in the cell can be performed by sodium. Granting that our knowledge of the function of potassium is imperfect, it seems likely that sodium can participate in the maintenance of the ionic balance in the cell sap. In the soil the presence of sodium might conceivably serve as a luxury supply of potassium if we assume, of course, that the minimum demands of the crop for potassium are met. This is suggested by the work of Lehr (32) on beets, who envisages an equilibrium condition between the cations, calcium, potassium, and sodium. Lehr concludes that sodium has "an almost independent function" in the foliage but not in the root. This does not define with precision whether the element is regarded by Lehr as essential or not. The concept of "stimulants" largely used by analogy with "stimulants" in animal physiology does not appear to the reviewer to be established on any sound foundation in plant nutrition.

Additional data on the boron-calcium relationship (see last year's review) have been presented by Marsh (33). A comparison has been made between the monocotyledons, maize and oats, and the dicotyledons, tobacco and faba beans. The level of soluble calcium in both types of plants was found to be determined not by the total calcium in the plant but rather by the boron content. When the fresh supply of calcium from the nutrient medium is cut off, the previously acquired calcium is maintained in an available (soluble) condition within the plant by an adequate boron content. Since the boron supply in the nutrient medium directly influences the boron content of the plant (for both mono- and dicotyledons), it may also be regarded as controlling the proportion of soluble calcium in the plant system. The boron in the nutrient medium, however, does not (within certain limits) significantly influence calcium absorption. The higher requirement of dicotyledons than monocotyledons for boron is explained by the smaller proportion of absorbed boron which remains in solution in the tissues of the former.

Another and, in many respects, exceptionally interesting interrelation of nutrients, that between iron and manganese, has been explored by Shive and his co-workers (34, 35). Many observations made on plants grown both in soils and in artificial nutrient media have repeatedly suggested to various investigators that the utilization of one of these metals by the plant is dependent on the other. Somers & Shive (34) investigated the interrelation between the two metals under carefully controlled conditions. They grew a series of soybeans

in basic nutrient solutions to which iron was supplied as ferrous sulfate at three different levels, 0.005, 0.5, and 3.0 p.p.m. At each iron level manganese was added as manganese sulfate at six different concentrations: 0.00,[4] 0.002, 0.01, 0.25, 2.0, and 5.0 p.p.m. The growth responses of the plants were found to be dependent on the relative proportion rather than on the absolute amounts of iron and manganese in the nutrient medium. Wide variations in the external supply of the two metals (0.005 p.p.m. iron to 0.002 manganese and 3.0 p.p.m. iron to 2.0 manganese) were compatible with good growth of plants and freedom from pathological symptoms as long as the ratio of iron to manganese fluctuated around 2.0. Ratios of iron to manganese above this range produced a chlorosis ascribed to iron toxicity, whereas ratios below this range resulted in symptoms of a different type of chlorosis attributed to manganese toxicity. The pathological symptoms traceable to an excess of iron were found to be the same as those caused by a deficiency of manganese, and, conversely, excess manganese gave symptoms analogous to those of iron deficiency. That is, manganese toxicity is equated with iron deficiency, and iron toxicity is the same as manganese deficiency.

Chemical determinations were made of iron and manganese in the plant and, as in the calcium-boron study, a distinction was made between the soluble and insoluble fractions. The soluble fractions of iron and manganese, those found in the expressed sap, were considered to be metabolically active, and the insoluble fractions, those found in the press cake, to be metabolically inert. Interestingly enough, it was found that a definite range of ratio values of soluble iron to soluble manganese was associated with good growth and freedom from chlorosis, and numerically it was almost coextensive with the effective range of external concentrations, 1.5 to 2.5. On the other hand, the ratios between total iron and total manganese in the plant showed no significant relation to plant growth. Low manganese in the nutrient medium corresponded to a low soluble manganese and a high soluble iron in the tissues. Conversely, high manganese in the external solution gave a high soluble manganese and a low soluble iron in the plant. High soluble iron in the plant was always associated

[4] This would imply that soybeans can be grown without any manganese in the nutrient solution. The reviewer assumes that the good growth reported from these cultures is accountable by the small contaminants in the nutrient medium in addition to the manganese reserves of the seed.

with iron toxicity and a high soluble manganese content with manganese toxicity symptoms.

Because of the probable participation of these two metals in cellular respiration, Somers, Gilbert & Shive (35) also measured the carbon dioxide production of the plants grown at the various levels of supply of manganese and iron. The highest yield of respiratory carbon dioxide was found in plants making good growth and free from chlorosis symptoms, which, as already mentioned, was coincident with an iron to manganese ratio in the substrate of around two. The absolute amounts of these metals, either within the plant or in the nutrient medium, were found to be without effect on the respiratory carbon dioxide.

Somers & Shive offer the following hypothesis to explain the observed relations. The cell can tolerate only a certain definite concentration of the physiologically active form of iron, which is of the ferrous valence. The function of manganese is to regulate the concentration of the ferrous iron in the cell. When the two metals are supplied in a balanced proportion, the excess of ferrous iron is oxidized by manganic ion into ferric iron which is precipitated in the form of "ferric-phosphorus organic complexes" and rendered physiologically inactive. A deficiency of manganese in the substrate leads to a deficiency of manganic ion in the plant and to a corresponding shift in the direction of a higher ferrous iron concentration and a resultant iron toxicity. On the other hand, an excess of manganese in the nutrient substrate is responsible for an excess of manganic ion in the plant and shifts the equilibrium in the opposite direction—that is, an excessive oxidation of ferrous iron and the subsequent iron deficiency synonymous with manganese toxicity.

It seems to the reviewer that the principal premise on which this hypothesis rests, that plants can utilize only ferrous iron, merits reexamination in the light of present views of iron function in living cells. The role of iron in oxidation-reduction systems, in which iron-porphyrin protein compounds such as the cytochromes or peroxidase take part, rests on the reversible oxidation-reduction of the iron, and hence both the ferrous and the ferric form of iron must be regarded as essential in metabolism. It is also open to question whether the physiologically active iron-porphyrin enzymes would be included in the expressed sap or the press cake fraction of the iron determination.

A relation between aluminum and copper was reported by Liebig,

Vanselow & Chapman (36). Low concentrations (0.1 to 0.5 p.p.m.) of aluminum were found to counteract copper toxicity in citrus cuttings grown in nutrient solutions. Higher concentrations of aluminum (2.5 to 5 p.p.m.) gave a curious stimulation of root growth accompanied by a depression of top growth. Spectrographic analyses of plants grown in nutrient solutions containing different concentrations of aluminum and copper indicated that the detoxifying effect of aluminum resided in the roots although the absorption of copper was not prevented. In the absence of aluminum, excessive copper caused a brownish appearance in citrus roots and short swollen laterals which gave the roots a dwarfed, knotty, and unhealthy appearance. Subsequent top growth often exhibited iron chlorosis.

High salt effects on plants.—It has been said that the history of civilization in arid regions contains many examples of failure which can be traced to the ultimate breakdown of the systems of agriculture based on irrigation. Aside from the contributing social and political factors, the failure of irrigated agriculture comes within the purview of plant physiology and soil chemistry since it often involves deleterious effects on crops by accumulated salinity in the soil.

An important landmark in the history of irrigated agriculture is the recent establishment by the United States Department of Agriculture of the Regional Salinity Laboratory at Riverside, California, which represents, to the writer's knowledge, the first organized scientific effort specially directed toward the understanding of the effects of high salt concentration on plant growth. Several contributions which have emanated from that laboratory in the past year will now be considered.

Eaton (37) grew a series of crops to maturity in outdoor sand cultures with variable equivalent concentrations of chloride and sulfate, the two principal anions of saline conditions in soils. Sulfate was found on the whole to be about half as toxic as chloride in the same concentration. For some plants the difference between the two ions was even more notable. Lemon plants were apparently four or more times as tolerant of sulfate as of chloride. But, on the other hand, beets withstood chlorides better than sulfates. Thus the results offer no grounds for evaluating the relative toxicity of the two anions on a simple physical or chemical basis. The growth-depression curves exhibited no evidence of an abrupt point at which toxicity effects become pronounced but showed that above some minimum concentration each successive unit of salt, considered by itself, tended to

produce a lesser depression in growth than the preceding unit. The symptomology of plants grown in saline substrates is perplexing in that while some crop plants such as barley, milo, and navy bean showed a leaf burn, a number of others, alfalfa, cotton, beet, and tomato, developed no symptoms of diagnostic importance.

Neither do the analyses of the expressed saps from the several species offer a direct basis for evaluating plant responses to high salt concentration in terms of ionic accumulation. On the whole an increase in the concentration of chloride in the substrate was paralleled by a corresponding increase in its concentration in the plant sap, but in the case of cations, the increments of their concentration in the sap were very slight in comparison with their increase in the nutrient medium. In harmony with Collander's (2) results, great variability was observed among the different plants in their capacity for sodium absorption. Pronounced differences among the species were also noted in case of sulfate absorption.

Although the root is the organ directly in contact with the saline substrate in which the plant grows, scant attention has been given heretofore to the effects of salts on roots. Hayward & Blair (38) have described the effects of varying chloride concentrations at two pH levels, 5.5 to 6.0 and 7.5 to 8.0, on the anatomical and physiological response of Valencia orange roots. They found that the anatomical response of the root to high chloride concentrations and high pH values resembles that of dormancy, a condition found normally in orange roots during which meristematic activity is diminished and suberization of epidermal cell walls takes place. These nutrient conditions, high chloride and high pH, were also associated with inhibition of growth in both shoots and roots, and chlorosis and tip burn of leaves. Few root hairs developed on roots grown under conditions of low hydrogen ion concentration and these were usually short or primordial in character.

A further inquiry into the mechanism of salt injury to higher plants was instituted by Gauch & Eaton (39), who investigated the effect of chloride and sulfate saline substrates on the hourly levels of carbohydrates and salt accumulation of barley plants. The plants were sampled at four-hour intervals over a twenty-four-hour cycle and analyzed for mineral constituents and carbohydrates. The results showed that the addition of both chloride and sulfate salts reduced in relation to the control plants (in a twenty-four-hour period) calcium and notably potassium concentration, left magnesium unchanged, and

brought about a marked increase in sodium concentration. Nitrogen and phosphate concentrations were little affected. Chloride was accumulated much more readily than sulfate ion. While the addition of sulfate to the nutrient medium markedly reduced chloride accumulation, the addition of chloride was without effect on sulfate accumulation. These absorption data taken jointly with those previously noted (37) emphasize the specific differences among plants in responses to saline conditions. As far as carbohydrates were concerned, the plants in the saline substrates showed a higher content of both sugars and starch suggesting interference of the accumulated salts with cellular activities associated with carbohydrate utilization.

Effect of the common cations of saline substrates, calcium and sodium, on salt accumulation by bean plants was considered by Gauch & Wadleigh (40). Dwarf red kidney beans were grown from the seedling to the flowering stage in nutrient solutions supplemented by a series of increasing concentrations of sodium chloride and calcium chloride solutions, respectively. Although both salts in high osmotic concentrations similarly restricted growth, they each influenced in a different manner the total absorption and distribution of the nutrient elements. The sodium chloride-plants absorbed larger total amounts of nitrogen, phosphorus, and potassium than did the calcium chloride-plants. The relative distribution of sodium and calcium in the aerial portions of the plant was relatively independent of the nutrient medium. Calcium predominated over sodium in the leaves and stems even in plants grown in the sodium chloride substrate. In the roots, however, sodium was markedly accumulated in the sodium chloride substrate without affecting their calcium content, which remained uniform throughout the plant. [Compare Collander (4).]

In attempting to account for the ionic relations within the plant, Gauch & Wadleigh suggested that plants absorbed hydrogen ions from the nutrient medium in order to balance the excess chloride accumulated in the leaves of the sodium chloride plants. Little is known about the absorption of hydrogen ions by the plant nor indeed has the very occurrence of it been demonstrated. (Obviously a technique using isotopes would be required for the study of hydrogen ion absorption.) It appears to the reviewer that the excess anion accumulation in the leaves can be explained on the basis of a decrease in organic acids, as demonstrated by Ulrich (6), and the subsequent release of bases to balance the chloride. Gauch & Wadleigh's figures indicate that, allowing for the elaboration of nitrogen into organic forms,

there were ample cation reserves in the leaf to balance the anions. The observed trend toward increased alkalinity in the sodium chloride solutions, which the authors also explain on the basis of hydrogen ion absorption by the plant, can likewise be accounted for by an exchange of the absorbed chloride with bicarbonate ions of metabolic origin.

The assimilation of nitrogen from sodium chloride and calcium chloride saline solutions of various osmotic concentrations was investigated by Wadleigh & Gauch (41). They found with the red kidney bean a linear decrease in green weight with an increase in the osmotic concentration of the substrate. Plants in the calcium chloride series made as much shoot growth (but less root growth) as those in the sodium chloride series. Increased salts in the nutrient medium were associated with decreases in the percentages of nitrate nitrogen and protein nitrogen in the plant. Protein nitrogen constituted a higher proportion of the assimilated nitrogen in the stems and roots of the calcium chloride series than of the sodium chloride series, and the authors suggest that these differences in nitrogen metabolism are related to specific effects of calcium and chloride ions on the hydration of colloids. The paper by Wadleigh & Gauch (41) suggests a comparison with the previously discussed work on nitrogen metabolism by Phillis & Mason (24).

Inorganic nutrients in relation to fruitfulness and nutritional quality of plant products.—The great advances of the last two decades in the field of animal and human nutrition have led to a growing preoccupation with the nutritional quality of agricultural crops. This new approach to problems of crop production in the field is reflected in the establishment by the United States Department of Agriculture of a Plant, Soil, and Nutrition Laboratory at Ithaca, New York, dedicated to the bridging of the gap between plant and animal nutrition. The growing concern with the nutritional quality of crops promises to provide a welcome balance to the heretofore dominant emphasis on yields.

One of the important questions of interest both to plant physiology and animal nutrition is the relation of environmental factors to the vitamin content of plants. Of the environmental complexes influencing plant growth, the one residing in the soil and including the supply of mineral nutrients affects the roots, and the other, involving chiefly climatic factors, affects the aerial portions of the plant. In the absence of accurate information the tendency in the past has often been to ascribe, in discussions of factors influencing the vitamin content of plants, a more important function to the soil than to the aerial environ-

ment. Hamner, Lyon & Hamner (42) from the Ithaca laboratory undertook the examination of this general problem by investigating the influence of climate and nutrition on the ascorbic acid content of tomato fruit. The general plan of their experiments provided for testing, on the one hand, a large number of nutrient combinations under the same set of climatic conditions and, on the other hand, growing plants under similar nutrient conditions in different localities and different climates. The nutrient conditions involved a wide range of combinations including a total deficiency of each of the six inorganic elements required by plants in large amounts: nitrogen, phosphorus, sulfur, potassium, calcium, and magnesium. With minor exceptions, their data showed no demonstrable effect of even large fluctuations in the supply of these six inorganic elements on the ascorbic acid content of tomato fruit, in treatments from which fruit was obtained. But the location and season during which the plants were grown were found to have a significant influence on the vitamin C content of the fruit. The conclusion was drawn that ascorbic acid in tomato fruit is significantly related to climatic rather than to soil conditions.

Similar conclusions concerning the importance of climatic factors on vitamin C synthesis were reached by Reid (43) who worked with young cowpea seedlings. She found the light factor to be particularly relevant. High daylight intensity and medium to long days were associated with high ascorbic acid content, whereas the reverse had a depressing effect. Light intensity was found to be of greater importance than length of day. Reid concluded on the basis of less extensive data that when light conditions permitted an ample carbohydrate synthesis, a sufficient nutrient supply with special reference to nitrogen was conducive to high ascorbic acid values. It is to be noted that this conclusion is not necessarily in conflict with the lack of correlation between nitrogen supply and ascorbic acid content of tomato fruit reported by Hamner *et al.* (42). Reid was concerned with short-time responses in young vegetative tissues and a nitrogen deficiency would interfere with the physiological activities of the plant, ultimately resulting in cessation of photosynthetic activity with the subsequent shortage of ascorbic acid precursors. The tomato fruit, on the other hand, would depend largely on organic reserves manufactured in the leaves.

Hamner *et al.* (42), as well as Lyon, Beeson & Barrentine (44), have reported observations on the effect of the various nutrient combinations on the growth, fruitfulness, and the occurrence of blossom-end

rot in tomato. They conclude, on the basis of a statistical analysis of results, that growth and fruitfulness could be correlated with relatively minor variations in nutrient compositions. The relation was particularly noted with variations in the amounts of calcium and nitrate in the nutrient medium. Lyon *et al.* (44) also found an increasing incidence of blossom-end rot with decreasing calcium concentrations in the nutrient medium. Chemical analyses of the fruit obtained from treatments in which the rot was most severe indicated that such fruits were low in calcium and high in potassium and magnesium content.

These conclusions invite comparison with some unpublished data from the writer's laboratory. It was found that if an adequate supply of nutrients was maintained, rather wide variations in the composition of the nutrient medium were without effect on the composition of the tomato fruit, which could only be influenced by rather extreme nutrient conditions. The differences in the experimental technique should be noted. Hamner *et al.* and Lyon *et al.* grew their plants in crocks in sand culture and had given them only an intermittent supply of nutrients by irrigating the sand several times a week with a nutrient solution, whereas in our experiments the plants were grown continuously in a very large volume of nutrient solution, in which the level of nutrients was continuously maintained within definite limits. It is possible that with the intermittent supply of nutrients, differences in concentration of the nutrient solution may in reality reflect differences in the total supply of a particular nutrient element, which can become critical in the minimum ranges.

The reported relation of calcium to blossom-end rot adds another experimental observation bearing on this problem. The reviewer is inclined on the basis of present evidence to favor the view that blossom-end rot is a general type of symptom reflecting a number of physiological disturbances all of which may result in a withdrawal of water from the fruit. Eaton (37) found the incidence of blossom-end rot in tomato associated with high salts in the nutrient solution, and indications from the recent experiments from the writer's laboratory, already referred to, suggest that blossom-end rot may be associated under certain conditions with phosphorus deficiency in the nutrient medium.

Oxygen effects on plants.—It would indeed be striking if the essential function of oxygen so firmly established in studies on mineral absorption were not reflected in favorable effects of aeration on growth.

There is general realization at the present time that an adequate oxygen supply constitutes an indispensable element of an artificial culture medium, and there are reasons to believe that it may often be a limiting factor in crop production in soil [Arnon & Hoagland (12)]. Evidence is being amassed which shows that while oxygen is essential for the growth of all plants, wide differences in quantitative requirements exist among various species. Taylor (45) has added to our understanding of the low oxygen requirement of rice as contrasted with wheat—a fact long evident from the different cultural practices under which these two crops are produced. Rice was found to possess a strong fermentation mechanism which is feeble in wheat. This explains why rice can germinate, and wheat cannot, in the absence of oxygen and also accounts for the ability of rice to accomplish with a low oxygen supply more nearly normal growth than wheat. But even rice cannot continue growing in the complete absence of oxygen. The aerobic respiration system, on the other hand, is weaker in rice than in wheat. Thus, wheat is able to utilize more effectively than rice a lower concentration of oxygen in respiration.

The quantitative oxygen requirements of three different species of plants, soybean, oat, and tomato, were investigated by Gilbert & Shive (46) by means of a specially adapted water-culture technique. Several oxygen levels ranging from 0 to 16 p.p.m. were maintained in the nutrient solution. The optimum growth of soybeans occurred in nutrient solutions containing 6 p.p.m. Below this optimum the plants showed decreased growth and abnormal root development. Above the optimal oxygen tension toxicity symptoms of abnormal leaf growth and browning of the roots were noted. The oxygen toxicity symptoms could be corrected either by lowering the oxygen tension or by adding ammonium nitrogen to the nutrient solution. They were aggravated by the presence of nitrate nitrogen. These findings on the relation of the form of nitrogen to oxygen requirements are in accord with the results of Arnon (47) with barley plants which led him to advance the view that nitrate nitrogen may, in addition to supplying nitrogen, serve as a source of oxygen upon reduction to an amino group within the plant. Tomato was again shown to have a high oxygen requirement; best growth was obtained with the highest oxygen level supplied, 16 p.p.m. Oats also were found to respond to oxygen in the nutrient medium particularly within the range of 0 to 4 p.p.m. They were also noted not to show oxygen toxicity symptoms at the highest oxygen concentration studied.

Diagnostic Methods in Assessing Nutrient Deficiencies

Several papers which appeared in the past year testify to the growing reliance on methods for assessing nutrient deficiencies that depend on physiological and chemical approaches based on the plant itself, rather than on the soil in which the plant grows. The plant obviously provides an integration of the exceedingly complex factors which determine the availability of nutrients in a soil. These methods illustrate the application and extension of principles of plant physiology and biochemistry to problems of great economic importance.

It goes without saying that any approach to nutrient deficiency diagnosis must take into account first the anatomical and physiological characteristics of the crop plant in question and second, the particular organ or product with which the culture of the plant is concerned. Many of the practical applications of this field of research, particularly as they pertain to specific crops and soils, are beyond the scope of this review. It is proposed to discuss briefly, however, some of the principles used in assessing nutrient conditions which are of general biochemical interest.

Nightingale (48) has reported extensive studies on the nutrition of pineapple in relation to fruitfulness. He was chiefly concerned with the development of a guide for nitrogen (nitrate) fertilization in the field with a view to bringing about maximum fruitfulness. His approach was to relate the need for nitrogen by the plant to the available supply of carbohydrates. Applied to pineapple the problem resolved itself to adjusting the concentration of nitrate to the starch reserves within the plant. Nitrate determinations were made on the white, semimeristematic tissue at the leaf base. As far as the estimation of starch content was concerned, Nightingale found that what mattered was not the absolute amount of starch in the plant, expressed on a percentage weight basis, but rather the total capacity for starch accumulation possessed by a plant at a particular stage of its development. For example, dark-green succulent plants containing actually a greater amount of starch than yellow-green woody plants but having in contrast with the latter a potential capacity for starch accumulation, were considered for diagnostic purposes as starch deficient. A workable correlation between the color of the leaf and its starch status was

devised: the greater the degree of yellow-green color of the plant, provided it did not approach senescence, the higher was its starch reserve appraised. By employing suitable standards of color, the relative greenness of a plant was quantitatively recorded and provided a sufficiently accurate index of its carbohydrate content. This starch determination taken jointly with the nitrate analysis provided a guide for fertilization. The purpose of nitrogen fertilization was to maintain an adequate nitrate reserve in relation to the carbohydrate supply. A balance between the two, of course, was essential for optimum fruitfulness.

Evans (49) compared two methods for determining nutrient deficiencies in sugar cane: the leaf and stem injection method of Roach (50) and the measurement of the growth increments in a selected number of plants fertilized *in situ*. The leaf injection method was found unsuited to the diagnosis of nutrient deficiencies in sugar cane, but the determination of fertilizer deficiencies from the growth responses of a selected number of plants actually growing in the field appeared to give in two months reliable guides as to fertilization procedure, with an accuracy at least equal to that of large-scale field trials. If the growth increments on the selected plants were obtained at the beginning of the period of active growth, the results were of use in guiding the fertilization of the standing crop.

Lorenz & Minges (51) studied the seasonal march of nutrient elements in lettuce by analyzing the sap of the midribs of the outer leaves. They were able to show that at the end of the growing season an abundant supply of nitrogen in the soil was reflected in a contraseasonal high concentration of nitrate in the plant sap. In field fertilizer experiments with potassium on grapes, Ulrich (52) compared the leaf analysis method with the determination of the replaceable and Neubauer potassium content of two soils as guides for supplying the potassium requirements of the plant. He found that the yields for three consecutive summers were directly related to the potassium content of leaf petioles but not to the measurements of soil potassium. The potassium in the petioles reflected the potassium status of the vines more accurately than that in the leaf blades. The leaves taken for analysis were the first "mature" leaves adjacent to the growing point. A similar correlation was found by Ulrich (53) between the nitrate content of the petioles of the most recently "matured" leaves and the nitrogen status of the vine. As in Nightingale's work (48)

nitrate nitrogen offered a reliable guide for appraising the total nitrogen status of the plant.

Ulrich bases his methods of foliar diagnosis on the assumption that

> for a given plant part found suitable for analysis, there is a minimum value (range) for each nutrient which characterizes a deficiency. Since there is in most instances only one dominant limiting nutrient or factor operating at a given moment, all other nutrients or factors are likely to be above the minimum value.

This point of view lays stress on the correction of minimum values indicative of a particular nutrient deficiency and eliminates the complex considerations of interrelations between several nutrients when present in concentrations above the minimum.

Present Status of Micronutrient Elements

Physiological aspects.—Steinberg (54), continuing his pioneering work on the utilization of micronutrient metals by *Aspergillus niger*, found that these, including gallium, were required whether inorganic nitrogen or amino acids were used as a source of nitrogen. In another paper (55) Steinberg presented several observations on the growth of *A. niger* when the micronutrient metals were omitted in the presence or absence of carbon dioxide. The omission of iron, zinc, and copper in the absence of carbon dioxide caused relatively greater decreases in the growth of the fungus than when carbon dioxide was present. A most interesting observation is the apparent effect of gallium, the omission of which, either singly or jointly with zinc, copper, or molybdenum, reduced the growth of *Aspergillus* in the absence of carbon dioxide much more than when carbon dioxide was present. It is to be hoped that more extensive data will be forthcoming to substantiate this observation. These responses were considered as probably associated with the phenomenon of dark fixation of carbon dioxide by the fungus—a process analogous to carbon dioxide fixation in the dark by higher plants.

Reed (56) investigated the relation of zinc to seed formation in peas, beans, and dwarf milo. For beans and peas a threshold value for the zinc was noted, below which only small seedless pods were formed. A zinc supply above the threshold value, even though suboptimal for growth, gave viable seeds. Likewise for milo, there was a threshold value of zinc for the development of tops and heads. In a

cytological study of plants deficient in zinc and other micronutrients, Reed & Dufrenoy (57) and Dufrenoy & Reed (58) observed spherical inclusions in cell vacuoles which they attributed to a condensation of the colloidal material normally distributed at random in the vacuole. They suggest that these aggregates which appear to consist of phenolic material (catechol) surrounded by a phospholipid boundary arise by a process of coacervation, caused by a change in equilibrium between the diffused colloidal material and the surrounding liquid.

The essential function of molybdenum and vanadium for nitrogen fixation by *Azotobacter* has been confirmed by Horner, Burk, Allison & Sherman (59). The effective concentration range for molybdenum and vanadium was found to vary from 1×10^{-5} or 1×10^{-4} p.p.m., which gave a detectable effect, to about 1 p.p.m., which gave maximum growth in six days. The view that both iron and molybdenum must be present in optimum concentrations for maximum growth and nitrogen fixation was also confirmed. Manganese, however, had no appreciable effect on *Azotobacter*.

The possible connection between the onset of manganese deficiency symptoms and the presence of micronutrients in the root environment was studied by Barker & Broyer (60). Some of the cultures were kept sterile, yet typical manganese deficiency symptoms with squash plants were obtained whether the roots were sterile or not. Plants heavily contaminated with soil microorganisms were less sturdy than the sterile ones, but this was true for both complete and manganese-deficient cultures. Such plants also demonstrated a higher respiratory rate of their excised roots than was found for comparable sterile roots. Barker & Broyer suggest that this may lead to a relative oxygen deficiency because of greater oxygen consumption and may be a factor in root injury.

Boron deficiency was found by Alexander (61) to be associated with increased catalase activity in the boron-deficient tissues. The greatest catalase activity was found on the whole in tissues which showed the earliest and most extensive biological symptoms of deficiency.

Among the numerous species of *Astragalus,* some have, but many have not the capacity to accumulate selenium and the accompanying usefulness as valuable indicators in locating seleniferous areas. Trelease (62) found that the selenium indicators can be distinguished from the nonindicator *Astragalus* species by a simple growth test of

seedlings in a culture solution containing selenium. The presence of 20 p.p.m. of selenium was well tolerated by the indicator species but it completely inhibited root growth in the nonindicator species. The absorption of selenium by corn plants from a nutrient solution was found by Trelease & Greenfield (63) to be influenced by organic substances of proteinaceous nature. The addition of 50 p.p.m. of bactotryptone, neopeptone, sodium caseinate, and proteose peptone approximately doubled the absorption of selenium from a solution containing 5 p.p.m. of selenium. Similar results were obtained from adding water extracts of alfalfa hay or string beans. It is suggested that selenium absorption by nonseleniferous plants may be enhanced by the presence of organic nitrogen in the soil.

Iodine toxicity to tomato plants within a range of 4 to 100 p.p.m. of iodine is reported by Hageman, Hodge & McHargue (64). Reduction in growth was not observed until a concentration of 16 p.p.m. was reached, but a decrease in ascorbic acid content and an increase in manganese and copper absorption were characteristic of all plants receiving iodine. It is to be noted that there is as yet no convincing evidence of the essentiality of iodine for plant growth.

List of essential micronutrients.—The essentiality of boron, manganese, and zinc for higher plants is no longer open to dispute, and limitations of space prevent the reviewer from listing the recent evidence of a confirmatory nature which extends our knowledge of the importance of these elements in the nutrition of different species of plants. There can also be little doubt that with proper experimental safeguards, the indispensability of copper can be consistently demonstrated. Piper (65) has recently confirmed the essentiality of copper for nine different species, and Hoagland (66) has shown the same for fruit trees. As for the lately proposed new essential element for higher plants, molybdenum (67, 68), recent evidence tends to establish its place on the list of indispensable nutrients. Piper (69) reported its need for oats, Hoagland (66) for Myrobalan plum, Bertrand (70) for leguminous plants, and Brenchley & Warrington (71) made recently a preliminary announcement of improved lettuce growth by the addition of molybdenum.

The question sometimes arises whether the list of essential micronutrients as it stands today is complete. The reviewer's opinion is that although a number of different species of plants have been grown successfully in highly purified nutrient solutions which supplied only

boron, manganese, calcium, zinc, and molybdenum, an unequivocal answer to this question cannot be given. It is certain that despite all caution, minute impurities were present in the nutrient medium. What can be asserted definitely is that if an element now regarded as dispensable for a given plant should at some future time be found essential, it will be shown to be required in exceedingly small amounts —within the limits of contamination still encompassed by the refined methods used.

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CARBON DIOXIDE ASSIMILATON IN HETEROTROPHIC ORGANISMS

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Earlier Work on the Participation of Carbon Dioxide in Cell Metabolism

Metabolic reactions of carbon dioxide.—Before 1936 it was generally assumed that the ability to assimilate carbon dioxide was a monopoly of autotrophic organisms. It had already been recognised, it is true, that carbon dioxide is not entirely inert in heterotrophic organisms: Carbon dioxide was known to combine with amino acids and certain proteins, e.g., haemoglobin to form carbamino compounds (1). It was generally accepted (2) [and has now been proved (3, 4)] that carbon dioxide serves as a source of carbon in the synthesis of urea. It was also known that a number of bacteria are capable of reducing carbon dioxide: In methane bacteria carbon dioxide reacts with the hydrogen of various organic compounds to form methane ($CO_2 + 8\,H = CH_4 + 2\,H_2O$) (5, 6); in various organisms of the coli group carbon dioxide and molecular hydrogen react to form formic acid ($CO_2 + H_2 = HCOOH$) (7). But these reactions were not looked upon as an "assimilation" of carbon dioxide because they do not lead to the incorporation of carbon dioxide into the cell structure. The formation of carbamino acids and carbhaemoglobin are nonenzymic processes which are entirely independent of the cell metabolism—though not necessarily without physiological significance (1). Urea, formic acid, and methane, as far as is known, are metabolic end products and one would therefore hesitate to call the formation of these substances from carbon dioxide "assimilation."

Effects of carbon dioxide on growth.—Perhaps the first indication that carbon dioxide plays an active part in the metabolism of heterotrophic organisms was the observation (Bang, 1897, Nowak, 1908) that carbon dioxide facilitated the cultivation of *Brucella abortus* (8, 9). By 1927 it was known that a number of other organisms, *Staphylococcus, Bacterium typhosum, Escherichia coli, Pseudomonas, Mycobacterium tuberculosis, Saccharomyces, Mucor, Asper-*

gillus (10, 11, 12), require carbon dioxide for their growth and the view was expressed "that the gas is used as a source of carbon" (10). Other workers, (13) notably Gladstone, Fildes & Richardson (14), confirmed and extended this work and showed that rapid removal of carbon dioxide from liquid cultures, by means of a continuous passage of carbon dioxide-free gases through the medium, entirely inhibits the growth of many bacteria. The authors concluded that "the presence of CO_2 in a medium was a pre-requisite for the growth of all bacteria examined," but they added "we have no theory to put forward to explain this phenomenon" (14).

Effects of carbon dioxide on cell metabolism.—Two further observations which indicated an active participation of carbon dioxide in metabolic processes were Elsden's discovery that the rate of succinic acid formation in *Escherichia coli* is dependent on the carbon dioxide pressure (15) and Hes's finding that the reduction of methylene blue by various microorganisms is inhibited when carbon dioxide is rigidly excluded (16). Viewed in the light of the present knowledge these observations appear highly significant, but at the time when they were made "facts were too few even to permit theorizing on the role of CO_2 in succinic acid formation" (15).

Carbon Dioxide Assimilation in Propionic Acid Bacteria

In 1936 Wood & Werkman (17), reporting experiments on the fermentation of glycerol by propionic acid bacteria, published the unexpected observation that in some experiments the end products of the fermentation (mainly propionic and succinic acids, with small quantities of acetic and lactic acids) contained more carbon than had been added to the medium in the form of glycerol. For instance, for 100 m*M* of glycerol (i.e., 300 m.eq. of carbon) fermented, 55.8 m*M* of propionic acid, 2.9 m*M* of acetic acid, and 42.1 m*M* of succinic acid (342 m.eq. of carbon) were recovered. As the medium contained calcium carbonate (to neutralise the acids formed during the fermentation) the authors suspected that this might have provided the excess carbon. In fact less carbonate was recovered than was originally present. In the experiment quoted the difference was 37.7 m*M* per 100 m*M* of glycerol fermented. This is very nearly the amount expected (42 m*M*). It was thus shown that propionic acid bacteria are able to utilise carbonate (or carbon dioxide) and to convert it into organic material.

In 1938 Wood & Werkman (18) extended their investigations

and showed, with improved methods of analysis, that the quantities of carbon dioxide utilised and of succinic acid formed are approximately equimolar. The data conformed with the assumption that there are two main reactions, each of a complex nature, when glycerol undergoes fermentation in propionic acid bacteria, namely:

(I) $\underset{\text{(glycerol)}}{CH_2OH \cdot CHOH \cdot CH_2OH} \rightarrow \underset{\text{(propionic acid)}}{CH_3 \cdot CH_2 \cdot COOH} + H_2O$

(II) $\underset{\text{(glycerol)}}{CH_2OH \cdot CHOH \cdot CH_2OH} + CO_2 \rightarrow HOOC \cdot CH_2 \cdot \underset{\text{(succinic acid)}}{CH_2 \cdot COOH} + H_2O$

Discussing the significance of the equivalence of carbon dioxide utilisation and succinic acid formation, Wood & Werkman considered two possibilities. They suggested

> that the formation of succinic acid is by synthesis from a 3-C compound through addition of CO_2; ... pyruvic acid .. may be the point of entry for CO_2.

Alternatively, they pointed out that

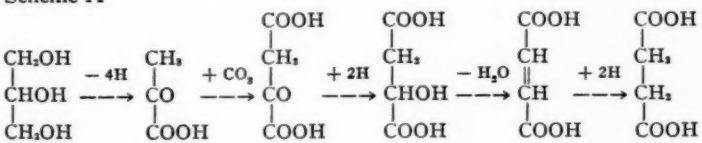
> The formation of the oxidized product, succinic acid, must be accompanied by a simultaneous reduction. Since the utilization of CO_2 involves reduction, the relationship of CO_2 and succinic acid may arise in the establishment of the oxido-reduction balance.

In 1940 Wood & Werkman (19) reported that propionic acid bacteria fix carbon dioxide not only with glycerol as the substrate, but also with mannitol, adonitol, erythritol, and rhamnose, and they gave their earlier suggestion of a "C_3 and C_1 addition" a more definite shape by stating

> A possible mechanism accounting for CO_2 utilisation as well as succinic acid formation involves the addition of CO_2 to pyruvic acid to form oxaloacetic acid, followed by reduction to malic acid, dehydration to fumaric acid and finally reduction to succinic acid.

(19). This concept is formulated in the following series of reactions:

Scheme A



This scheme, like others, must be regarded as a simplification requiring further elaboration. It has been suggested, e.g., that phosphorylations occur during the fermentation (19). Krebs & Eggleston

(20) found that the postulated reactions readily occur in propionic acid bacteria: Oxaloacetic acid is reduced to fumaric and malic acids; fumaric and malic acids are reduced to succinic acid. Furthermore, fumaric acid was shown to be formed during the fermentation of glycerol (20). The fact that propionic acid bacteria are equipped with the enzymes which bring about the series of reactions A may be regarded as an indication that these reactions are normal intermediary processes in propionic acid bacteria. Moreover, if it is borne in mind that the rate at which oxaloacetic acid can be reduced to succinic acid is relatively rapid and that therefore oxaloacetic acid if synthesised by "carboxylation" of pyruvic acid is bound to undergo immediate reduction; the detection of the reduction products can be considered as proof of the formation of oxaloacetic acid, especially as no alternative mode of formation of fumaric and succinic acids is known.

Further progress in the elucidation of the problem was due to the introduction of isotopes of carbon. Carson & Ruben (21) employed the radioactive isotope, C^{11}, whose rapid decay (half-life, twenty-one minutes), however, limits the scope of its application. Wood, Werkman, Hemingway & Nier (22, 23) used the stable isotope, C^{13}. It was found in confirmation of scheme A that the succinic acid form contained the carbon isotope used to label the carbon dioxide added and that the carbon assimilated was present in the carboxyl groups of the succinic acid.

Unexpectedly, propionic acid was also found to contain the utilised carbon in its carboxyl group (22, 23). This result indicated that the mechanism of the propionic acid fermentation of glycerol was not as simple as had previously been assumed. To explain the occurrence of fixed carbon in propionic acid the following mechanism was suggested (20, 24):

$$\text{glycerol} \rightarrow \underset{\downarrow}{\text{pyruvic acid}} \overset{+\,CO_2}{\underset{-\,CO_2}{\rightleftharpoons}} \text{oxaloacetic acid} \rightleftharpoons \text{malic acid} \rightleftharpoons \text{fumaric acid} \rightleftharpoons \text{succinic acid}$$
$$\text{propionic acid}$$

This scheme comprises only reactions which have been shown to occur in propionic acid bacteria. Its main feature is the assumption that pyruvic acid can be converted reversibly into a symmetrical dicarboxylic acid (fumaric acid). Pyruvic acid undergoing these reactions would contain carbon dioxide carbon, and on reduction of pyruvic acid the assimilated carbon would appear in propionic acid. Werk-

man & Wood (25) argue that it is difficult to reconcile with this scheme the quantities of fixed carbon found in propionic acid, for the concentrations of fixed carbon in the carboxyl groups of propionic and succinic acid were found to be almost equal (25). Therefore, if the above scheme is correct, practically every molecule of propionic acid formed from glycerol would have to be converted first into fumaric acid. In other words, propionic acid will not be formed by reduction of pyruvic acid before the latter passes through fumaric acid and back again, and this Werkman & Wood consider unlikely. Moreover,

> if there was a rapid shifting back and forth from pyruvate to fumarate, part of the dicarboxylic acids should contain fixed carbon in both carboxyl groups, since some pyruvic acid containing fixed carbon would be present and could re-enter the fixation reaction.

In fact it seems, though it is not certain, that the succinic acid formed from glycerol contains all the fixed carbon in one carboxyl group (25). Werkman & Wood, whilst pointing out these difficulties, admit that they have no alternative explanation to offer and they agree that in spite of the difficulties it is probable that the decarboxylation of a symmetrical dicarboxylic acid, possibly of succinic acid, plays a part in the formation of propionic acid. Meyerhof (26) and Slade *et al.* (27) consider it possible that there is a nonenzymic interchange of hydrogen and hydroxyl between the α- and β-carbon atoms of enol-oxaloacetic acid or phospho-enol oxaloacetic acid, viz.,

$$HOOC \cdot CH = C(OH) \cdot COOH \rightleftharpoons HOOC \cdot C(OH) = CH \cdot COOH.$$

So far no experimental evidence has been adduced in support of this idea; if it is correct, it would no longer be necessary to assume a reversible formation of fumaric acid from oxaloacetic acid.

The presence of carbon, from the carbon dioxide in propionic acid, clearly indicates that the three-carbon chain of glycerol undergoes some fundamental rearrangement during the fermentation. Such a rearrangement may be associated with, or necessitated by, a phosphorylation, which may be brought about by the following series of reactions (28, 29):

Scheme B

pyruvic acid + CO_2 → oxaloacetic acid
oxaloacetic acid → fumaric acid
fumarate + phosphate + O → phosphopyruvate + CO_2

Some evidence in support of this set of reactions has been discussed by Lipmann (28) and by Solomon *et al.* (29). Whilst it cannot be claimed that these reactions have been proved to occur in propionic acid bacteria, the hypothesis indicates how the supposed difficulties might be overcome.

Carboxylation of Pyruvic Acid in Other Bacteria

The assimilation of carbon dioxide by way of carboxylation of pyruvic acid probably occurs in numerous microorganisms. The fact that the reaction was first discovered in propionic acid bacteria is due to the circumstance that most microorganisms produce carbon dioxide from added substrates and thus mask a simultaneous uptake, whilst in propionic acid bacteria reactions producing carbon dioxide are absent when glycerol is anaerobically fermented. The tracer method is, therefore, in many cases the only means of proving the assimilation of carbon dioxide.

Succinic acid produced under various conditions, as shown in Table I, has been found to contain isotopic carbon. Again this was located in the carboxyl groups. It is, therefore, most probable that the mechanism of succinic acid formation in these bacteria is the same as in propionic acid bacteria. In the case of *Escherichia coli* the conclusion is further supported by the accelerating effect of carbon dioxide on the rate of succinic acid formation (15) and by the observation that the synthesis of succinic acid is accompanied by formation of fumaric and malic acids (20).

Succinic acid formed from hexoses also contains carbon derived from assimilated carbon dioxide in the carboxyl group (27). This result seems to dispose of those hypotheses of fermentation which, in order to explain the formation of succinic acid, have assumed a fission of sugar into two compounds, one containing four carbon atoms and the other two carbon atoms (32).

Whether all the succinic acid formed by fermentation of sugars, glycerol, or pyruvic acid, and related substances arises *via* the carboxylation of pyruvic acid is an open question. Werkman & Wood (25) believe that a second mechanism is possible, viz., by condensation of two molecules of acetic or pyruvic acids. The writer (20) is of the opinion that the facts known so far are in accordance with the assumption that the carboxylation of pyruvic acid is the only mechanism whereby the four-carbon chain arises from pyruvic acid.

Bacterial Formation of Lactic and Acetic Acids Involving Carbon Dioxide

As will be seen from Table I, isotopic carbon has also been found in lactic acid and in acetic acid formed by various bacteria under conditions where labelled carbon dioxide was assimilated. In every case the labelled isotope was located in the carboxyl groups. The occurrence in lactic acid of carbon derived from carbon dioxide can be explained on the same lines as its occurrence in propionic acid, i.e., on the assumption that a symmetrical four-carbon dicarboxylic acid is an intermediate precursor of lactic acid.

Various possible mechanisms of entry of carbon dioxide into the carboxyl of acetic acid have been discussed by Slade *et al.* (*27*). Acetic acid is generally believed to arise by oxidation of pyruvic acid, but if the latter contains isotopic carbon in the carboxyl group, the resulting acetic acid would be devoid of such carbon. Slade *et al.* consider it possible that the initial fixation of carbon dioxide takes place through the carboxylation of pyruvic acid and that acetic acid is derived from a four-carbon dicarboxylic acid by a fission of the molecule into two two-carbon fragments. At the same time, the authors discuss alternative explanations based on the assumption that the carboxylation of pyruvic acid is the reaction by which carbon is fixed (*27*).

Total Synthesis of Acetic Acid from Carbon Dioxide in Bacteria

Whilst it is possible that in the organisms so far considered the carboxylation of pyruvic acid is the only process by which carbon dioxide is assimilated, it is certain that in another group of heterotrophic microorganisms carbon dioxide is fixed in a different way. Wieringa (33) observed that sporeforming organisms from mud belonging to the clostridium group are able to convert carbon dioxide and molecular hydrogen quantitatively into acetic acid. Barker, Ruben & Beck (34) found that *Clostridium acidi urici* forms from 1.1 to 1.25 molecules of acetic acid from one molecule of hypoxanthine. The quantity of acetic acid was thus greater than can be obtained directly from the three-carbon chain of hypoxanthine and this indicates that part or all of the acetic acid was synthesised from carbon dioxide or other one-carbon fragments of the purine. Using the tracer method, Barker, Ruben & Beck (34) confirmed this conclusion

TABLE I

LOCATION OF ASSIMILATED CARBON DIOXIDE IN MICROORGANISMS

Organism	Substrate	Compounds in which isotopic carbon, added as labelled CO_2 or $NaHCO_3$ has been found	Compounds in which no isotopic carbon has been found	References
Propionibacterium pentosaceum	glycerol	succinic acid propionic acid propyl alcohol		(23, 31)
Propionibacterium pentosaceum	glucose	succinic acid		(23)
Escherichia coli	pyruvic acid	succinic acid	ethyl alcohol acetic acid	(23, 31)
Escherichia coli	galactose	succinic acid	acetic acid	(23, 31)
Escherichia coli	pyruvic acid	fumaric acid malic acid aspartic acid		(30)
Escherichia coli	glucose	fumaric acid		(30)
Aerobacter indologenes	glucose	succinic acid lactic acid acetic acid formic acid	ethyl alcohol 2,3-butylene glycol	(27)
Streptococcus paracitrovorus	citrate	succinic acid	lactic acid acetic acid	(27)
Streptococcus paracitrovorus	glucose	succinic acid lactic acid	ethyl alcohol acetic acid butylene glycol	(27)
Clostridium welchii	glucose	acetic acid lactic acid	butyric acid ethyl alcohol butyl alcohol acetone	(27)
Clostridium acetobutylicum	corn mash	lactic acid	acetic acid butyric acid ethyl alcohol butyl alcohol acetone	(27)
Proteus vulgaris	glucose	succinic acid lactic acid	acetic acid ethyl alcohol	(27)
Staphylococcus candidus	glucose	succinic acid lactic acid	acetic acid ethyl alcohol	(27)

and located carbon dioxide carbon in the carboxyl as well as in the methyl group of acetic acid.

The distinguishing feature of carbon dioxide assimilation in this group of organisms is the formation of a carbon chain in which two neighbouring carbon atoms are derived from carbon dioxide. The carboxylation of pyruvic acid, even if followed by a variety of secondary reactions, can hardly be expected to lead to the formation of such a chain and it follows that carbon dioxide is fixed in these organisms by reactions which are different from, though perhaps analogous to, the carboxylation of pyruvic acid. A possible, if entirely speculative, mechanism is the following:

$$CO_2 \xrightarrow{+4H} HCOH \xrightarrow{+CO_2} HCO \cdot COOH \xrightarrow{+4H} CH_3 \cdot COOH$$

Carbon Dioxide Assimilation in Moulds

The work on the formation of the four-carbon dicarboxylic acid in bacteria (18, 19) and of citric acid in animal tissues (35, 35a) and the fact that carbon dioxide facilitates the growth of moulds (12, 36) made it appear probable (18) that carbon dioxide fixation, by way of carboxylation of pyruvic acid, also plays a part in the metabolism of the moulds which form di- or tricarboxylic acids as end products. Foster, Carson, Ruben & Kamen using C^{11} as a tracer were the first to support this view with direct experimental proof (37). Since moulds grown in stationary liquid cultures produce thick pellicles on the surface which, owing to their physical and physiological inhomogeneity, are unsuitable for many metabolic studies, moulds were cultivated in a flask placed on a rotating shaking machine [cf. Kluyver & Perquin (38)], a procedure which yields a submerged growth in a homogeneous suspension. A strain of *Rhizopus nigricans* which forms fumaric acid from glucose was grown in 5 per cent glucose saturated with carbon dioxide. The fumaric acid formed was found to contain the labelled isotope of carbon, and this was located exclusively in the carboxyl groups. In addition, assimilated carbon was found in the acid-insoluble cell material (probably protein). A strain of *Aspergillus niger* known to produce citric acid from sugar was found to yield a citric acid containing the tracer carbon in the carboxyl groups.

The fact that carbon dioxide takes part in the formation of citric acid in *Aspergillus* throws fresh light on the mechanism of this synthesis (25, 37). Chrzaszsz (39) and Bernhauer (40) had earlier sug-

gested (1930, 1934) that sugar first undergoes an alcoholic fermentation, that the alcohol thus formed is oxidised to acetic acid, and that the latter subsequently yields citric acid by a series of condensations in which four-carbon dicarboxylic acids are intermediates [see (39)]. In 1936 Wells, Moyer & May (41) showed that these schemes are incompatible with the high yields of citric acid from sugar under some conditions. The maximum yields expected according to the schemes of Chrzaszsz and of Bernhauer were 1 molecule of citric acid for 1.5 molecules of sugar fermented and 3 molecules of carbon dioxide formed. Wells *et al.* found that the formation of 1 molecule of citric acid required no more than 1.21 molecules of glucose and was accompanied by the formation of only 1.09 molecules of carbon dioxide. Similar yields were observed independently by Raistrick & Clutterbuck (42), and by Butkewitch & Gajevskaja (43). At the time when these observations were made it was not possible to offer a satisfactory explanation for the high yields. As a result of the recent work a hypothesis accounting for the yields can be outlined: The first stage is an incomplete alcoholic fermentation, yielding two three-carbon compounds which undergo oxidation to pyruvic acid. Whilst one molecule of pyruvic acid is decarboxylated yielding acetaldehyde and probably on further oxidation acetic acid, a second molecule of pyruvic acid is carboxylated to oxaloacetic acid. Citric acid may then arise by way of a condensation reaction either between oxaloacetic and acetic acids [Raistrick & Clark (44)] or malic and acetic acids [Chrzaszsz (39)]; acetic acid may react in the form of acetyl phosphate [Lipmann (28)]. A condensation between acetaldehyde or pyruvate and a dicarboxylic acid must also be considered as a possibility. Thus this conception, whilst defining the major features of the formation of citric acid, leaves a number of alternatives open with regard to details. One of the possible pathways which can be easily adapted is summarised by the following scheme:

$$\text{glucose} + 2O \rightarrow 2 \text{ pyruvic acid} + 2H_2O$$
$$\text{pyruvic acid} + O \rightarrow \text{acetic acid} + CO_2$$
$$\text{pyruvic acid} + CO_2 \rightarrow \text{oxaloacetic acid}$$
$$\text{oxaloacetic acid} + \text{acetic acid} \rightarrow \text{citric acid}$$

The net effect of these four reactions (or of the modifications considered above) is:

$$\text{glucose} + 3O = \text{citric acid} + 2H_2O$$

Thus the theoretical maximum yield of citric acid is one molecule per molecule of glucose, and no carbon dioxide is evolved in the synthesis.

The fact that the observed yields are smaller and that some carbon dioxide is formed can be explained by the assumption that the synthesis of cell material (which always occurs concurrently with the synthesis of citric acid) entails some loss of substrate and involves the formation of carbon dioxide.

Carbon Dioxide Assimilation in Animal Tissues

Carbon dioxide "assimilation" in animal tissues was discovered in 1940 (35a, 45, 46, 47), four years after it had been established in propionic acid bacteria. The observations which led to the discovery of carbon dioxide assimilation in animal tissues were made independently of the work on bacteria, and at a time when the mechanism of the bacterial assimilation was still obscure. But without the knowledge of carbon dioxide assimilation in heterotrophic bacteria, the idea that carbon dioxide assimilation occurs in animal tissues, and might be looked for, would hardly have suggested itself.

Pigeon liver.—The discovery of carbon dioxide assimilation in animal tissues arose from studies of the fate of pyruvic acid in pigeon liver. This tissue was found to be capable of synthesising a five-carbon chain from added pyruvic acid: Örström *et al.* (48) showed that ammonium pyruvate is converted into glutamine. As ammonium α-ketoglutarate and glutamic acid also yielded glutamine, it was thought that these two substances might be intermediate stages in the synthesis of glutamine from ammonium pyruvate (48). Subsequently, it was shown by Evans (49) that α-ketoglutaric acid is in fact formed in considerable quantities when sodium pyruvate is added to suspensions of respiring pigeon liver. At this time it had already been reported (50) that various animal tissues can synthesise α-ketoglutaric acid when pyruvic acid and a four-carbon dicarboxylic acid are added, the intermediate stages being assumedly the following:

Scheme C

$$\text{C}_4\text{dicarboxylic acid} \xrightarrow{+\text{O}} \text{oxaloacetic acid} \xrightarrow[-\text{CO}_2]{+\text{pyruvic acid} + \text{O}}$$

$$\text{citric acid} \xrightarrow{-\text{H}_2\text{O}} \textit{cis}\text{-aconitic acid} \xrightarrow{+\text{H}_2\text{O}}$$

$$\text{isocitric acid} \xrightarrow[-\text{CO}_2]{+\text{O}} \alpha\text{-ketoglutaric acid}$$

In contrast to other tissues (e.g., muscle) pigeon liver is capable of forming α-ketoglutaric acid without added oxaloacetic acid. Evans

(49), therefore, came to the conclusion that α-ketoglutaric acid can arise in pigeon liver in two ways, first according to scheme C, and second by way of an unknown mechanism not requiring a four-carbon dicarboxylic acid. It is now clear that the "second" mechanism, occurring in pigeon liver, differs in one step only from the first: Pigeon liver, unlike muscle, is capable of synthesising oxaloacetic acids from pyruvic acid and carbon dioxide and can therefore dispense with the supply of a four-carbon dicarboxylic acid.

The observation which provided the clue to the mechanism of the reactions taking place in the presence of pyruvic acid in pigeon liver was the discovery that the formation of α-ketoglutaric acid is always accompanied by a synthesis of *l*(-)malic acid and of smaller quantities of citric and succinic acids (35a). The simultaneous appearance of these substances might at first be taken as a matter of course since they are known to be interconvertible when oxygen is available. But the acids also arise in the absence of molecular oxygen, i.e., under conditions where the oxidative conversion of α-ketoglutaric acid into malic acid is not possible. But even when oxygen is present, the amount absorbed is too small (under certain conditions) to account for a primary synthesis of α-ketoglutaric acid and subsequent oxidative formation of the other acids.

When these facts came to light it was already known that pigeon liver forms α-ketoglutaric, malic, citric, and succinic acids when oxaloacetic acid is added anaerobically (51). Moreover, the proportions in which these substances appear are almost the same with oxaloacetic and pyruvic acids though the rate is more rapid with oxaloacetic acid. This highly significant fact is shown in Table II.

A simple explanation for the fact that oxaloacetic and pyruvic acids yield the same products is offered by the assumption that pyruvic acid can be carboxylated to oxaloacetic acid. To prove the correctness of this assumption it would be ideal to demonstrate the removal of pyruvate and carbon dioxide and the formation of stoichiometric quantities of oxaloacetic acid. As it happens, however, oxaloacetic acid is one of the most reactive substances among the intermediary metabolites in animal tissues: One milligram (dry weight) of pigeon liver (52) or pigeon breast muscle (33) can metabolise anaerobically 0.011 mg. of oxaloacetic acid in one minute (not counting the spontaneous decomposition to pyruvic acid and carbon dioxide). This is about ten times as much oxaloacetic acid as may be expected, on account of the yields of α-ketoglutaric, citric, fumaric

and malic acids, to arise under similar conditions from the carboxylation of pyruvic acid. Hence, the prospects of a successful isolation of the oxaloacetic acid formed by carboxylation of pyruvic acid are slight, if not nil. There remains the alternative method of identifying oxaloacetic acid from its derivatives. "Derivatives" of oxaloacetic acid are the substances which arise from oxaloacetic acid in tissue, viz., α-ketoglutaric acid, fumaric acid, malic acid, citric acid, and succinic acid.

TABLE II*

ANAEROBIC METABOLISM OF PYRUVIC ACID AND OXALOACETIC ACID IN PIGEON LIVER

Substrate	Pyruvic acid	Oxaloacetic acid
Substrate used	890 μl.	1344 μl.
Metabolites formed:		
succinic acid	45 μl. (yield 5.1 per cent)	61 μl. (yield 4.5 per cent)
α-ketoglutaric acid ..	131 μl. (yield 14.7 per cent)	197 μl. (yield 14.6 per cent)
fumaric acid + malic acid	334 μl. (yield 37.6 per cent)	580 μl. (yield 43.1 per cent)
citric acid.....	12 μl. (yield 1.3 per cent)	11 μl. (yield 0.8 per cent)

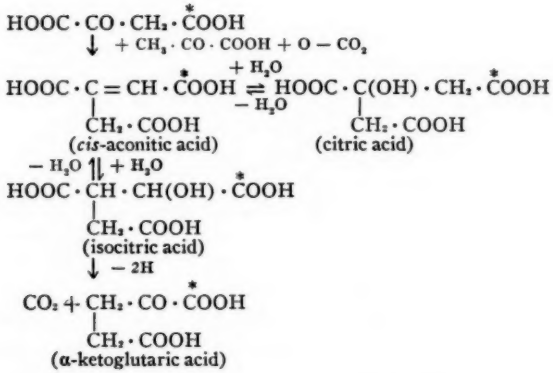
* The data for pyruvic acid are taken from Krebs & Eggleston (35a), those for oxaloacetic acid from Evans (49). The yields are expressed as percentage of substrate used. Period of incubation: 60 minutes in the pyruvic acid experiment, 40 minutes in the oxaloacetic acid experiment. The figures refer to 4 ml. of liver suspensions containing 400 mg. of tissue (wet weight). 1 μl. $= \frac{1 \text{ millimole}}{22{,}400}$ mg. substance.

Whilst the formation of one or two of the substances may not be specific for oxaloacetic acid, the complete combination of the five substances in definite proportions, as shown in Table II, must be regarded as specific for oxaloacetic acid, for no other substance (except pyruvic acid) is known to give this combination anaerobically. In the view of the writer, the conclusion that pyruvic acid is converted into oxaloacetic acid is therefore inescapable. This conclusion is further supported by the observation that the rate of pyruvic acid removal and of α-ketoglutaric acid formation is dependent on the concentration of bicarbonate and carbon dioxide (35a).

Such doubt as remained concerning the validity of this argument arose from the entirely novel feature of an "assimilation" of carbon dioxide in animal tissues. These doubts were disposed of by experiments with carbon isotopes carried out by Evans & Slotin (45, 46) and Wood, Werkman, Hemingway & Nier (47). These experiments

demonstrated directly the participation of carbon dioxide in the synthesis of α-ketoglutaric acid and of malic acid. As expected, the fixed carbon was found in the carboxyl groups of the dicarboxylic acids. Whilst taken alone, this result does not prove the occurrence of the carboxylation of pyruvic acid as Evans & Slotin (46) pointed out; nevertheless, the cumulative force of the available evidence is, in the view of the writer, conclusive.

The further analysis (46, 47) of the tracer experiments showed that, within the limits of the accuracy of the method, all the fixed carbon in α-ketoglutaric acid was present in the carboxyl group adjacent to the keto group. This is not compatible with the intermediary mechanism of α-ketoglutaric acid formation formulated in scheme C. According to this scheme, fixed carbon should be found in both carboxyl groups of α-ketoglutaric acid since one of the intermediates, citric acid, is a symmetrical molecule. Wood *et al.* (47) have pointed out that a minor modification of scheme C—a different order of formation of the tricarboxylic acid—as shown in the following scheme,[1] accounts for this observation:


HOOC · CO · CH₂ · *COOH
+ CH₃ · CO · COOH + O − CO₂
+ H₂O
HOOC · C = CH · *COOH ⇌ HOOC · C(OH) · CH₂ · *COOH
− H₂O
CH₂ · COOH
CH₂ · COOH
(cis-aconitic acid)
(citric acid)
− H₂O
+ H₂O
HOOC · CH · CH(OH) · *COOH
CH₂ · COOH
(isocitric acid)
− 2H
CO₂ + CH₂ · CO · *COOH
CH₂ · COOH
(α-ketoglutaric acid)

For a more detailed discussion of this subject the reader is referred to the papers of Werkman & Wood (25, 47) and of Krebs (52).

Lactic acid formed in pigeon liver on addition of pyruvic acid also contains carbon dioxide carbon in the carboxyl group (47). This can

[1] The asterisk in the following formulae denotes the carbon atom derived from assimilated carbon dioxide.

be explained by the mechanism already discussed for the case of the formation of isotopic propionic and lactic acids in bacteria (47).

Glycogen synthesis in rat liver.—Solomon *et al.* (29) injected intraperitoneally into rats solutions of sodium bicarbonate containing radioactive carbon, C^{11}, and fed lactate orally at the same time. Glycogen formed in the liver was found to contain radioactive carbon in quantities varying from 7 to 16 per cent of the carbon. These yields indicate that up to one carbon atom out of six of a glucose molecule can be derived from carbon dioxide. The authors suggest that the entry of carbon dioxide may be through the carboxylation of pyruvic acid, followed by the reactions depicted in scheme B. These would yield phosphopyruvic acid, whence glycogen can be synthesized by a reversal of the reactions of glycolysis. This explanation is, of course, hypothetical as the evidence supporting scheme B is incomplete. An attractive feature of the scheme is the fact that it explains the incorporation of carbon dioxide into the di- and tricarboxylic acids and into glycogen by one and the same primary reaction, the carboxylation of pyruvic acid.

Succinic acid formation in protozoa.—In 1935 Reiner, Smythe & Pedlow (54) observed that *Trypanosoma lewisi* decomposes glucose anaerobically by forming, among other products, succinic acid. The maximal yield of succinic acid was one molecule per molecule of glucose fermented. In 1940 (55) Searle & Reiner extended this work, guided by the newer knowledge of the mechanism of biological succinic acid formation, and found, by examining the carbon balance, that carbon dioxide is absorbed by the organism. The amount of assimilated carbon, as in the case of the propionic acid bacteria, was found to be equivalent to the amount of succinic acid formed. Furthermore, the dissimilation of glucose and of glycerol was found to be accelerated by the addition of carbon dioxide or bicarbonate to the medium. It is obviously suggestive to assume that the mechanism of carbon dioxide assimilation is the same as in other organisms, but Searle & Reiner point out that the fact that pyruvic acid does not seem to undergo any change in this organism argues against this assumption. The authors discuss the formation of glucose carbonate and glycerol carbonate.

Another protozoon has been studied by van Niel *et al.* (56) who report that the holotrichous ciliate, *Tetrahymena geleii,* forms lactic, acetic, and succinic acids and absorbs carbon dioxide when sugar is fermented anaerobically. With the aid of C^{11} the assimilated carbon

was located in the carboxyl groups of succinic acid (and possibly of fumaric acid).

Creatine synthesis.—From the work of Borsook & Dubnoff, Schoenheimer, and du Vigneaud [reviewed by Mitchell (57)] it is now known that creatine arises in the animal body from glycine, arginine, and methionine. Glycine and arginine interact to form glycocyamine which is methylated by methyl transfer from methionine:

$$\text{glycine} \xrightarrow[\text{(in kidney)}]{\text{+ guanidino group of arginine}} \text{glycocyamine} \xrightarrow[\text{(in liver)}]{\text{+ methyl group of methionine}} \text{creatine}$$

The arginine required in the kidney for this synthesis may come directly from the proteins of the food. Alternatively it can arise from ornithine, citrulline being an intermediate [see (58)]. Since the carbon atom of the guanidine group of arginine formed in this way is derived from carbon dioxide and since the creatine synthesised according to the above scheme contains this carbon atom, carbon dioxide carbon may be expected to be present in creatine and glycocyamine. So far this has not been tested experimentally.

Occurrence of the carboxylation of pyruvic acid in various animal tissues.—Pigeon muscle does not form measurable quantities of α-ketoglutaric acid from pyruvic acid under the conditions that this occurs in pigeon liver (35, 46). So far pigeon liver and pigeon muscle are the only tissues which have been examined with the tracer method. A synthesis of the C_5 chain (glutamine) from pyruvic acid has been found in duck and fowl liver (48) and in small quantities in guinea pig kidney, brain, cortex, and liver (52, 59).

Distribution of Assimilated Carbon in Heterotrophic Organisms

Only a relatively small number of substances has so far been directly shown to contain assimilated carbon, but owing to the central position of oxaloacetic acid in intermediary metabolism it is to be expected that its synthesis by carboxylation of pyruvic acid results in the appearance of assimilated carbon in many of the substances synthesised by heterotrophic cells and tissues. In animal tissues, oxaloacetic acid can be converted into citric, isocitric, *cis*-aconitic, α-ketoglutaric, malic, fumaric, and succinic acids; by reductive amination of α-ketoglutaric and oxaloacetic acids, glutamic and aspartic acids and hence protein-containing fixed carbon can be formed; on

the basis of scheme B, or an analogous set of reactions, one would expect fixed carbon dioxide in pyruvic acid, alanine, lactic acid, and glycogen; by way of the glycolytic breakdown of glycogen, fixed carbon would enter the intermediaries of the Embden-Meyerhof scheme. As carbohydrate can be converted into fat, assimilated carbon probably also finds its way into fat. In those bacteria which can synthesize virtually all cell constituents from lactic acid, it is likely that assimilated carbon is present in many substances. In the last two cases the incorporation of fixed carbon into organic compounds is at present only a matter of conjecture as the mechanisms of the synthesis of fat and of bacterial cell constituents are unknown; it is possible that fixed carbon is lost in the interconversion process.

Number of Primary Fixation Reactions

The observations on animal tissues and on the bacteria (with the exception of *Clostridium acidi urici* and Wieringa's organism) can be explained on the assumption that the carboxylation of pyruvic acid is the only reaction by which carbon dioxide is fixed to a carbon chain. This, of course, does not imply that no further fixation reactions occur, but at present it is unnecessary to postulate the occurrence of several mechanisms of fixation.

Relations between Photosynthesis and Heterotrophic Carbon Dioxide Assimilation

Ruben & Kamen (60), van Niel *et al.* (61), and Franck & Gaffron (62) discuss the question whether the reactions by which carbon dioxide can be fixed in heterotrophic organisms also play a role in photosynthesis. Evidence is adduced to support the assumption that the primary process in photosynthesis is a conversion of carbon dioxide into a carboxyl group, by way of a reaction somewhat analogous to the carboxylation of pyruvic acid ($RH + CO_2 \longrightarrow R \cdot COOH$) and that this is followed by a photochemical process in which the carboxyl group is reduced to alcohol ($R \cdot COOH + H_2O + nh\nu \xrightarrow{\text{chlorophyll}} R \cdot CH_2OH + O_2$). This hypothesis predicts that the fixation of carbon dioxide (because it precedes the photochemical reaction) can occur in the absence of light. In fact, green algae were found to fix isotopic carbon dioxide in the dark (60). A considerable portion of the assimilated carbon dioxide was located, as postulated, in carboxyl groups.

Carbon Dioxide as a Growth Factor

The fact that carbon dioxide is required by growing organisms of many different types—bacteria, protozoa, moulds (12, 16, 63)—may be attributed to need of carbon dioxide for metabolic processes. The widespread occurrence of the carbon dioxide effect on growth suggests that carbon dioxide takes part in some fundamental process common to many organisms. Although it is by no means certain that the carboxylation of pyruvic acid is the only process in which carbon dioxide is needed, this reaction is sufficiently general and fundamental to offer an explanation for the indispensability of carbon dioxide. If the carboxylation of pyruvic acid is the only reaction requiring carbon dioxide, it should be possible to replace carbon dioxide in the culture medium by malic, fumaric, or succinic acids, or asparagine, or other substances which can serve as a continuous source of oxaloacetic acid. It seems that this has not been experimentally tested.

Biological Significance of the Carboxylation of Pyruvic Acid

The significance of carbon dioxide assimilation through carboxylation of pyruvic acid obviously depends on the biological significance of the oxaloacetic acid formed by this reaction. So far two fundamental functions of oxaloacetic acid are known: It serves as a parent substance of a number of essential cell constituents (as already discussed) and it plays a role as a catalyst in cell metabolism (56, 64).

Hydrogen transport by oxaloacetic acid was discovered in 1935 by Szent-Györgyi (65), who observed that oxaloacetic acid is rapidly reduced to malic acid in respiring tissues and that malic acid can undergo rapid reoxidation to oxaloacetic acid. The system, oxaloacetic acid $\underset{-2H}{\overset{+2H}{\rightleftharpoons}}$ malic acid (and in some cases the system, oxaloacetic acid $\underset{-4H}{\overset{+4H}{\rightleftharpoons}}$ succinic acid) seems to be an essential link in the respiration of animal tissues, and of a number of bacteria. It also appears to be operative in certain anaerobic fermentations, e.g., the glycerol fermentation of propionic acid bacteria (20), or the dismutation of pyruvic acid in *Staphylococcus* (66). The appearance of succinic acid among the products of fermentation of sugars and three-carbon compounds in many organisms may be taken to indicate that oxalo-

acetic acid is synthesised in the organisms concerned and that the reversible oxidation and reduction of the four-carbon dicarboxylic acid is a component reaction of the energy-giving mechanism. Some authors, however, believe that succinic acid can also be formed through condensation of the molecules of acetic (or pyruvic) acid and that the appearance of succinic acid does not necessarily indicate a carboxylation of pyruvic acid (35).

As already mentioned, it has been suggested by Lipmann (28) and others (29) that hydrogen transport by four-carbon dicarboxylic acid may be coupled with phosphorylations. The chief basis for this assumption is Kalckar's observation that the oxidation of fumaric acid in kidney extract results in the formation of a phosphorylated compound which is believed to be phosphopyruvic acid (67).

A further, no less important, catalytic function attributed to oxaloacetic acid is that formulated in the modified citric acid cycle (now called "tricarboxylic acid cycle") in animal tissues [see (52)].

The facts so far available suggest that the significance of carbon dioxide assimilation, by way of carboxylation of pyruvic acid, lies in the fact that this reaction is an essential link in the synthesis of indispensable cell constituents.

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BIOCHEMISTRY OF MICROORGANISMS

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A striking shift in emphasis has occurred in this field. Only a few years ago studies in general metabolism, especially carbohydrate decomposition, were by far the most numerous. The sound approach to the problem of microbial nutrition, initiated especially by Lwoff (1) and Knight (2), has led to such evident successes that studies of this kind now occupy an important place. This all the more since they have a direct bearing on the nutrition of higher organisms; many growth factors for microorganisms have been identified with typical vitamins (3).

Another fashionable subject is that of the bacteriostatic and bacteriolytic agents. Last year Dubos[1] could devote an entire review to this phase. The numerous new papers warrant a brief discussion of important advances.

Though these phases are obviously related to the metabolic activities of microorganisms, their study has contributed little to a better understanding of the chemical mechanisms involved in the breakdown of foodstuffs and synthesis of cell materials. Few attempts have yet been made to follow up the important leads (Lwoff, Fildes, Knight) in elucidating the mode of action of vitamins with vitamin-deficient cultures. On the other hand, advances have been made in the methods of preparation of enzyme fractions from microorganisms.[2] The importance of studies with such materials for a comprehension of metabolic processes can hardly be overrated in spite of Nord's admonitions (4). So far no convincing evidence has ever been presented to justify the conclusion that the isolated steps uncovered with separated enzyme systems do not occur in the normally metabolizing organism although there is little doubt that for a complete picture of the chemical events of metabolism a consideration of such factors as spatial orientation and coordination of special structures will also be required (5).

The use of manometric methods has, understandably, not dimin-

[1] *Ann. Rev. Biochem.*, **11**, 659–78 (1942)

[2] *Ibid.*, **10**, 553–86 (1941)

ished. But it is necessary to re-emphasize the occasional difficulties in the interpretation of results. It is often taken for granted that the oxygen utilization or gas production by cell suspensions in the absence of added substrate continue unaffected by the addition of metabolites. Yet it has been clearly shown that often the "endogenous" metabolism is inhibited when an external substrate is present (6, 7, 8). If the endogenous activity is small compared with the rate of decomposition of added metabolites, it matters little in the final analysis whether or not a correction is applied. Nevertheless, in order to make an evaluation possible, it is urged that the actual data for both processes be published rather than just the "corrected" figures.

Some most important contributions have been made to the problem of carbon dioxide utilization by autotrophic bacteria. These results should, of course, be integrated with those obtained in the study of carbon dioxide reduction by photosynthetic and by heterotrophic organisms. Since both photosynthesis and carbon dioxide assimilation by heterotrophic organisms are dealt with in special chapters in this volume, such treatment will be omitted here. [See also (9, 10, 11).] Furthermore, in view of the previous policy of the editors to publish regularly special sections on biological nitrogen fixation, this topic has been left out here while a treatment of the biochemistry of the fungi has been scheduled for next year's volume.

Growth Factors of Microorganisms

Nutrient requirements.—Four general papers (12 to 15) have been published since Barker's review.[3] Little can be added to Williams' excellent survey of growth factors for yeasts. That various strains differ is again emphasized by studies on osmophilic *Zygosaccharomyces* species (16). All are reported to need biotin, a few inositol, none pyridoxin or, surprisingly, thiamin. Pantothenic acid, in every case replaceable by β-alanine, is required for growth by some strains, stimulates that of others, but has no effect on the rest. The importance of amino acids for yeast growth (17), the effect of the amount of inoculum, time of harvesting, etc. (18) show that a simple interpretation of results with complex media is still difficult. It has also been stated (19) that *Saccharomyces cerevisiae* can be trained, by massive transfers in media deficient in one component at a time, to grow in the absence of factors originally needed.

[3] *Ann. Rev. Biochem.*, **10**, 553–86 (1941)

The use of yeast strains for assays of biotin, pryidoxin, inositol, and thiamin has been well established. While the Texas group (20, 21) uses growth as a basis for computation, modifications, particularly for thiamin determinations, have been based upon metabolism measurements (22 to 26). Important is a method which allows a separate determination of thiamin and cocarboxylase by fermentation rates (27). The response of yeast to inositol is specific (28, 29).

Synthesis of vitamins by yeast has long been known, especially in connection with the culture of *Torula utilis* on wood sugar and other industrial wastes. An extensive study of the addition of thiamin, thiazole, and pyrimidine at various stages during development on the thiamin content of yeast (30) has revealed the possibility of producing cells with up to 6 mg. of thiamin per gm. under carefully controlled conditions. Upon prolonged incubation a decrease is observed, and the highest conversion of added thiazole and pyrimidine into thiamin is obtained after most of the sugar has been utilized. Also, the addition of "spent medium" along with the thiazole and pyrimidine to nongrowing yeast results in a considerable thiamin synthesis; phosphate, peptone, or yeast extract are inferior to the "spent medium." These facts suggest that the synthesis proceeds during the oxidation of small amounts of alcohol present in the spent medium and that thiamin might be partly decomposed during sugar decomposition. (See later discussion of the "wearing out" of phosphopyridine nucleotides.)

Many algae can grow in the light in purely mineral media. However, their development can be greatly enhanced by the addition of organic substances, and the use of media with meat or soil extracts, sugar, etc. has often been recommended. Added to some studies on the mineral nutrition (31, 32, 33) and one investigation in which a striking increase in the yield of *Chlorella* is described following the addition of a small amount of malate to sugar-containing media, while in the absence of sugar the malate has little effect (34), is a curious report (35) on the effect of ascorbic acid and thiamin on the growth of *Haematococcus pluvialis*. The former vitamin appears as the limiting factor for growth in mineral media in the light; in dark cultures (acetate as carbon source) thiamin acts in such manner; in illuminated acetate media both the vitamins show a pronounced effect. Whether these phenomena are more general remains to be determined, as is also their relation to the observation (36) that *Chlorella* cultures produce substances which inhibit their further development.

For some of the green flagellates, even when illuminated, organic growth factors have been held necessary for some time. Dusi has now obtained completely autotrophic development of *Euglena anabaena* and *Eudorina elegans* (37, 38). This requires the addition of the proper minerals in carefully adjusted concentrations. Of especial interest is the requirement of iron salts for colony formation in *Eudorina*. Also for the colorless flagellates *Polytoma* and *Polytomella* the iron requirements have been stressed (39). *Euglena gracilis* has been cultured in darkness in the absence of vitamins (40) just as its colorless counterpart, *Astasia*, had previously been shown to grow under similar conditions (41). *Oikomonas termo*, another colorless flagellate recently obtained in pure culture (42), has been grown in media with glucose or glycerin and an organic nitrogen source. From the fact that any one of three different amino acids supports development, it may be inferred that its minimum requirements are simple though a much more copious growth can be obtained with peptone or yeast extract.

That flagellates grown in the absence of vitamins would produce vitamins during development seems a logical assumption. Hitherto tested only with bacteria, yeasts, and molds, this hypothesis has received support from the demonstration that the colorless *Chilomonas paramecium*, cultured in a mineral medium with acetate, contains 0.8 μg. of diphosphopyridine nucleotide per mg. of cells (43).

The growth requirements of ciliate protozoa are far more complex, and little accurate work has so far been possible since this necessitates studies with pure cultures [see the reviews by Kidder (44) and Hall (45)]. The successful establishment of such cultures of *Colpoda* (46, 47) and *Paramecium* (48) has opened the way for more intensive work. Already, thiamin, pyridoxin, and nicotinic acid have been identified as the important constituents supplied by yeast extract for the growth of *Colpoda* (49). The conclusion that protein degradation products of a special size are necessary for *Colpidium campylum* (50), because proteose but not bacto-peptone can be used for growth, appears premature; these products differ in far more respects.

Advances in our knowledge of the vitamin requirements of various bacteria include the identification of *p*-aminobenzoic acid as the unknown factor needed, in addition to biotin, for growth of *Clostridium acetobutylicum* (51, 52). This acid had previously been reported (53) as the only one required for this organism. This result

should probably be ascribed to biotin contamination of the medium since Lampen & Peterson's later results have been fully confirmed (54). Biotin has now been recognized as an essential growth factor for a hemolytic streptococcus (55), for some strains of *Rhizobium* (56) and of *Staphylococcus aureus* (57), and for the *Brucella* group (58, 59). *Bacillus larvae* appears to need only thiamin (60).

It is almost a foregone conclusion that a newly discovered growth factor, needed in minute amounts by some microorganisms, will soon be shown to be a vitamin for higher organisms (3, 61). This has been true for *p*-amino benzoic acid (62), which has furthermore been established as a growth factor for *Acetobacter suboxydans* (63) and some lactic acid bacteria (64, 65). These organisms have been recommended for assay methods (66, 67). By way of exception, pimelic acid, a growth factor for *Corynebacterium diphtheriae* (15), had not been identified with a vitamin, and its simple structure is not conducive to ascribing a coenzyme activity to the molecule. The gradual elucidation of the configuration of biotin and the characterization of its unbranched fatty acid chain (68) led to the idea that pimelic acid might be used by some organisms for biotin synthesis. With *Coryneb. diphtheriae* du Vigneaud *et al.* (69) showed a strikingly similar behavior of biotin and pimelic acid, while Eakin & Eakin (70) found that *Aspergillus niger* responds to the addition of pimelic acid to the medium not by more extensive or rapid growth, but by increasing its biotin content about twentyfold. Whether oleic acid, so strikingly effective in increasing growth of *Coryneb. diphtheriae* (71) and of *Erysipelothrix* (72), may also function in this or in a similar manner remains to be determined. Often, the need for biotin seems to imply more than the inability of the organism to synthesize pimelic acid since this compound could not satisfactorily be substituted for biotin in the case of thirteen molds known to require vitamin H (73).

Nucleotides as growth factors have been announced for certain lactic acid bacteria, where both purine and pyrimidine bases are essential (74, 75), and for *Clostr. tetani,* which seems to require only a purine in addition to numerous known vitamins (76, 77). Cozymase is not quite specific as a growth factor for *Hemophilus parainfluenzae;* it can be replaced by acid-treated dihydrocozymase and even by the desamino derivative (78). This led to the important discovery that from nicotinamide nucleoside, in which the linkage between the amide and pentose is preformed, the organism can synthesize the nucleotide. With a mixture of nicotinamide, adenylic acid, and *d*-ribose, the actual

carbohydrate constituent of coenzymes I and II (79), no growth is obtained. Thus the highest degree of specialization in biosynthesis of these coenzymes so far observed requires only one further chemical linkage than in most mammals and some microbes.

The extensive use of bacteria for vitamin B_2 assays has brought out some complications as yet imperfectly analyzed. While the Snell & Strong method (20, 21) has been found satisfactory for cereals by Wright (80), others have shown that the presence of starchy materials leads to values which are too high (81 to 85). Previous hydrolysis with enzyme preparations has been recommended to overcome the difficulty. Modifications have also been suggested for nicotinic acid (86 to 88), pantothenic acid (89 to 93), biotin (94), and phosphopyridine nucleotide assays (95, 96). The use of bacteria for estimating ascorbic acid where this might be present as the dehydrocompound (97) looks satisfactory although elsewhere experiments have indicated that similar organisms cause a destruction of this vitamin (98). The use of ascorbic acid to facilitate the culture of anaerobic organisms (99) probably depends upon the strong reducing quality of the substance.

It remains difficult to interpret the studies on amino acids as nutrient factors for bacteria (see also Barker[2]). Lactic acid bacteria may be stimulated or inhibited by amino acid mixtures (100, 101), but it is not possible to ascribe the effects observed to any one particular component. The same has been observed with *Salmonella* species (102, 103) and strikingly with luminous bacteria (104).

Microbial synthesis of vitamins.—The synthesis of undetermined growth factors has been reported for various soil bacteria (105, 106); in the latter case there is evidence to show that the growth factors are in part different from those commonly associated with such materials as yeast extract. More specific cases are the synthesis of: riboflavin by *Staph. aureus* (107), *Asp. niger* (108, 109), *Eremothecium* (110), and, possibly, by *Candida albicans* (111); a substance with ascorbic acid properties, also in its clinical effects, by *Serratia* from xylose (112), large amounts of biotin by *Phytomonas tumefaciens* (113). Of especial interest is the study on the B vitamins produced by the completely autotrophic *Thiobacillus thiooxidans* (114) in which thiamin, riboflavin, nicotinic and pantothenic acids, pyridoxin, and biotin have been detected: evidence that the organism is fully equipped with the regular complement of B vitamins and cannot well be considered as possessing a "primitive" type of metabolism. A number of intestinal

bacteria also form biotin, riboflavin, thiamin, and nicotinic acid, appreciable amounts being found in the medium (115). A more complete and quantitative study of such phenomena (116) has been carried out with five representatives (*Aerobacter aerogenes, Serratia marcescens, Pseudomonas fluorescens, Proteus vulgaris,* and *Clostr. butylicum*), all grown in a medium with 0.1 per cent hydrolyzed casein, 2 per cent glucose, and a supplement of minerals. Only for *Clostr. butylicum* was added 1 μg. biotin per liter of solution. The cultures were analyzed after twenty-four hours, separate assays being run on the cells and the medium. There is remarkably little variation in the vitamin content of the different bacteria; the only striking exceptions are furnished by the pantothenic acid and pyridoxin content of anaerobically grown *Aerobacter* cells which are two to three times as high as for the same strain grown aerobically or for the other bacteria. The real differences exist in the vitamin content of the media. In general *Ps. fluorescens* ranks highest as a producer of B vitamins; only nicotinic acid production is spectacular in the case of *Clostr. butylicum* (4.3 times that of the next highest, an aerobic, and 29 times that of the lowest, an anaerobic culture of *Aerobacter*), while pantothenic acid is formed most abundantly by *Aerobacter,* both under aerobic and anaerobic conditions (from 2 to 20 times that of the others). Special experiments on the rate of biotin accumulation in the medium as related to the rate of growth of *Proteus* showed convincingly that excretion is not due to autolysis of the cells. Another interesting result is that the addition of any one of the B vitamins in excess does not seem to affect the synthesis of it nor of any of the others by *Aerobacter.* Apparently different in this respect are the reactions of yeast to added thiamin (30) and of *Lactobacillus casei* to pyridoxin (117), which appears to be stored in quantities far greater than needed for maximal growth. In this connection it may be pointed out that the three homofermentative species of lactic acid bacteria tested all require pyridoxin while the heterofermentative types included can synthesize it.

Function of growth factors.—Quastel & Webley believe to have assembled evidence for the view that thiamin functions in the oxidation of acetate by propionic acid bacteria (118, 119). Apart from the fact that the organisms used are certainly not *Propionibacterium* species—they grow rapidly, aerobically, and on plain peptone agar! Krebs & Eggleston (120) also found that this strain does not carry out a propionic acid fermentation—there are certain aspects of the research which are hard to fit in with previous experience. For example, it is

stated that in the oxidation of acetate the molar ratio of oxygen consumption to acetate utilization is 1.90 to 1.99. This implies a complete oxidation of the substrate. But it has been shown repeatedly, with a wide variety of organisms, that in manometric experiments with resting cells acetate oxidation does not require more than 1 to 1.5 moles of oxygen per mole of substrate (121). Furthermore, it is hazardous to deduce from the observed Q_{O_2}'s the far-reaching conclusions that have been drawn. A single instance may make this clear. Suppose that the oxidation of lactate passes through pyruvate with its further decomposition being the limiting factor, one should then find a Q_{O_2} for lactate twice as high as for pyruvate, which has actually been observed provided that the data are not "corrected" for endogenous respiration. Important is the demonstration that bacteria without added components of the thiamin system (thiamin, potassium and magnesium ions) can be activated by previous immersion in solutions containing these substances, and that sodium hexosediphosphate can replace potassium. The experiments with a combination of two substrates are again difficult to interpret because their initial decomposition might require different enzymes while leading to common intermediates acted upon by enzyme systems not completely saturated by either substrate. In such a case, oxidation rates can be observed ranging from the rate for one of the substrates to a rate completely additive for the two compounds. In connection with experiments on the oxidation of acetate in the presence of oxaloacetate, the authors remark that a catalytic effect of the latter is possible because occasionally a more than additive result is obtained. This, however, is true only if the observed Q_{O_2}'s are corrected which, in view of the high metabolic rates, is probably unjustified. That simultaneous oxidations of two substrates undoubtedly occur, follows from the fact that the rate of disappearance of pyruvate is decreased by the presence of propionate, indicative of the operation of a common enzyme system fed by two reactions at a rate greater than commensurate with its maximum capacity. The numerous data are thus hard to evaluate. They do not exclude the possibility that in the acetate metabolism keto acids function which require the more commonly accepted mediation of carboxylase and hence of thiamin.

Krebs had postulated that carboxylase functions primarily in catalyzing the synthesis, from pyruvate and carbon dioxide, of oxaloacetate which is required for internal hydrogen transport (122). In support he claims that propionic acid bacteria can be grown aerobically

in air with 5 per cent carbon dioxide. This the author has not been able to verify; if slants are heavily inoculated aerobic development is possible, but in that event development occurs equally well in air, and a differential effect of increased carbon dioxide content has, in numerous cases, not appeared. With thiamin-deficient cells of *Staph. aureus* and *albus,* Smyth (123) has followed another approach. In the decomposition of pyruvate such cells respond equally to the addition of thiamin and of oxaloacetate or fumarate. While this evidence supports Krebs' hypothesis, no such effects have been observed when tested with thiamin-deficient cells of *Prototheca* (E. H. Anderson, personal communication). Results of Barron & Lyman also fail to support the idea (124), as do those of Krampitz & Werkman (310).

Addition of nicotinic acid to *Salmonella* suspensions increases the rate of oxidation of sugar, lactate, and acetate (125). In *Shigella* nicotinamide evokes a prompt response, nicotinic acid a delayed response (126). Hence it is probable that the latter is first converted into the former. Diphosphopyridine nucleotide is more effective than the triphospho-compound, and the activity of both is considerably increased by hydrolysis. The respiration of *Proteus,* however, is stimulated as readily by coenzyme I as by the hydrolyzed product or by nicotinic acid. So far, these results can still be explained conventionally by assuming that the various derivatives ultimately function as phosphopyridine nucleotides for which *Shigella* would be less permeable than *Proteus.* Further results with *Proteus* cells, grown in sugar and suspended in lactate or glutamate, are not so easily reconciled with this simple concept, for such suspensions are not activated by nicotinic acid, its amide, hydrolyzed phosphopyridine nucleotides, or intact coenzyme II, but only by coenzyme I. Saunders *et al.* assume the formation of another enzyme from the split products and find support for this in the observation (127) that inhibition of *Proteus* by pyridine-3-sulfonic acid is counteracted more by nicotinamide than by coenzyme I, from which they conclude that nicotinic acid may act otherwise than as a building block for coenzymes I and II. The remarkable study of Morel (128) shows, however, that this is not necessarily so. She found that the growth phase exerts a profound influence on the response of *Proteus* to nicotinamide. During the logarithmic phase the dehydrogenase activity per cell remains constant, and the metabolism of the organisms is not affected by added vitamin. After the inflection point the reducing power decreases by about 15 per cent per hour, and for some time the full activity can be restored by nico-

tinamide addition. With increasing lapse of time such restoration becomes more and more difficult. Then even centrifuging and washing of the cells causes further, apparently irreversible loss of dehydrogenase activity.

Using *Hemophilus* as a test organism, Morel could also show the gradual disappearance of coenzyme II from *Proteus* cells utilizing sugar, which is ascribed to a metabolic "wearing out." With cells adapted to pyruvate and metabolizing this substrate, only coenzyme I disappears, and its content can be kept up by supplying nicotinamide. It is thus concluded that the coenzymes only wear out while functioning due to an occasional irreversible change of the amide. From the rates of wear and the total number of oxido-reductions carried out by each enzyme molecule, it is computed that such "accidents" occur approximately once in every 200,000 reactions. This investigation is one of the finest examples of a purposeful study of the function of vitamins in metabolism. Perhaps the inactivation of pyridine nucleotides by animal tissues (129, 130), specifically inhibited by nicotinamide, is due to similar events.

Two noteworthy attempts have been made to elucidate the function of pantothenic acid in metabolism. Dialyzed yeast juice does not show an effect of pantothenic acid addition on the rate of glucose fermentation, the phosphorylation of sugar, or the decomposition of pyruvate, whereas its addition to living yeast, grown with a limiting amount of the vitamin, results in an accelerated rate of gas production (131). Meanwhile, the pantothenic acid is converted into a "bound" form. It does not, therefore, function directly as a dissociable coenzyme for systems involved in the sugar fermentation. The oxidation of sugar, and especially of lactate, by pantothenic acid-deficient cells of *Proteus* is also stimulated by this growth factor while dried cells, still capable of oxidizing lactate, fail to respond (132). These dried cells do not attack pyruvate which accumulates during lactate oxidation; fresh material produces acetate as an intermediate product whose further oxidation is stimulated by pantothenic acid, though to a much smaller extent than that of lactate.

Pyridoxin may well be converted by various organisms into a far more active compound. Snell *et al.* (133) have noticed the appearance of a "pseudopyridoxin," some 6,000 times more active than pyridoxin, in animal tissues exposed to an excess of vitamin B_6. Curious is the fact that lactic acid bacteria require pyridoxin but apparently do not use it. If transformation into a much more active

compound occurred, it is not clear why such a transformation would stop when practically all the pyridoxin is still present, or else why the relatively large doses, as compared with "pseudopyridoxin," are required.

Antibacterial Agents

Sulfonamides.—Woods' hypothesis that sulfanilamide and related substances owe their bacteriostatic effect to the structural similarity with *p*-aminobenzoic acid, with which they can compete at enzyme surfaces, has been reviewed (134, 135, 136) and corroborated by further studies on the antisulfonamide effect of *p*-aminobenzoate with bacteria (137 to 140), molds (141), protozoa (142), and diatoms (143). An alternative hypothesis has been proposed (144) but severely criticized (145, 146), and it must be recognized that the attempts to produce new bacteriostatic agents as analogues of known growth factors have resulted in products whose activity is, on the whole, so well in agreement with expectation (147, 148, 149) that the fruitfulness of the original concept has been established. Special mention should here be made of the pantothenic acid analogues (150, 151, 152). These possess activity only for organisms which require pantothenic acid as growth factor, and their bacteriostatic effect is inhibited by pantothenic acid itself. Certain tryptophane analogues whose inhibitory effect can be overcome by tryptophane also fall in this group (152a).

An influence of the sulfonamides on metabolism has been looked for by Sevag *et al.* (153 to 156) with positive results although these results are as yet too general to permit a closer analysis. The conclusion has been drawn that sulfonamide inhibitions result primarily from a blocking of the respiratory enzymes which, in turn, inhibit growth. It seems likely, however, that this view must be abandoned because it has been shown (157) that there exists no parallelism between inhibition by sulfonamides of respiration and of growth; the most striking example was that the respiration of a sulfonamide-sensitive and of a sulfonamide-resistant strain was equally inhibited.

Meanwhile, precise determinations of the dissociation constants of the sulfonamides have been made (158, 159), and from a comparison of the activities of the representatives at different pH values it is clear that the anions are responsible for the effects. In agreement with this conclusion are the findings that the sulfonamides affect the cataphoretic mobility of bacteria in a manner which associates their func-

tion with the amino group (160). A most satisfactory and comprehensive picture as to the relative activities of the various members has been developed on the basis of physico-chemical considerations (161). Here it may be pointed out that this concept makes it clear that sulfapyridine and sulfathiazole would act as *p*-aminobenzoic acid derivatives rather than as pyridine or thiazole analogues as seems implied in some papers (162).

It has, nevertheless, been found that substances other than *p*-aminobenzoate, and without structural similarity to it, such as methionine, xanthine, guanidine may also block the sulfonamide effects (162 to 167). It is quite evident that many interrelations exist between actual or potential growth factors and the drugs. Some of these are reminiscent of the amino acid antagonisms mentioned before; others show a more clear-cut picture. A few examples may be given. By frequent transfers to media containing sulfanilamide and some amino acids, including methionine, *Escherichia coli* has been transformed into a strain which requires methionine for growth even in the absence of sulfanilamide, while control cultures, either in amino acid-containing media without or in mineral-glucose media with sulfanilamide, grow normally upon transfer to a mineral-glucose medium (165). Hemolytic streptococci which require adenine as growth factor are not inhibited by sulfonamide in the presence of adenine (166). For species of lactic acid bacteria which grow only when supplied with purine bases these substances also counteract the sulfonamide effects (167). From all these observations it appears logical to subscribe for the present to the view that the drugs inhibit anabolic reactions such as the synthesis of methionine.

Gramicidin, penicillin, etc.—McIlwain has formulated the most general hypothesis as to the mode of action of chemotherapeutic agents (168).

> A chemotherapeutic agent is regarded as depriving the inhibited organism of the use of enzymes or metabolites by various types of interference. The organism thus becomes nutritionally more exacting than in its normal state, and its new demands can, with due consideration for extraneous effects, be analysed by the usual techniques of bacterial nutrition.

The mechanism of action of antibacterial agents produced by aerobic sporeforming bacteria (169 to 172),[4] actinomycetes (173, 173a, 174, 175), myxobacteria (176 to 179), and molds (see below), is, with one

[4] *Ann. Rev. Biochem.*, **11**, 659–78 (1942)

exception, quite unknown. Effects on metabolism by gramicidin have been observed (180), but an interpretation cannot yet be attempted: staphylococci have been made resistant (181), Gram-negative bacteria sensitized to the substance (182), the latter by the addition of protamines which presumably interact with phospholipids, and a stabilizing effect is exerted by tyrothricin on tetanus toxin (183).

Some of the antibacterial substances from molds have been prepared chemically pure (184, 185, 186) while conditions for their production and methods for purification and assaying have been developed (187 to 191). The most powerful samples of penicillin—which can inhibit sulfonamide-resistant Gram-positive bacteria (192)—have been analyzed; the formulas $C_{14}H_{19}NO_6$, $C_{24}H_{32}O_{10}N_2Ba$, and $C_{24}H_{34}O_{11}NSr$ represent the results, respectively, for the free acid with 240 (193), the barium salt with 450 to 500 (194), and the strontium salt with up to 750 Oxford units per mg. of substance (195). It seems probable that the single nitrogen in the last formula is due to a misprint; analytical data are not published in the paper. That growth in the presence of penicillin temporarily continues, unaccompanied by cell division and followed by lysis (196), was confirmed (197), but the mechanism of its action is still unknown (198, 199).

Notatin, a product of *Penicillium notatum,* appears to be a glucose dehydrogenase (200), a "yellow" dinucleotide enzyme which exerts anti-bacterial activity for *Staph. aureus* in dilution of $1:10^9$ when both glucose and oxygen are present and catalase is absent. The fact that penicillin inhibition is not affected by exudates seems to preclude that its mode of action is similar.

The inhibitory effect of human saliva is due to at least two factors (201). A crystalline protein or polypeptide from wheat flour (202) which affects growth of bacteria and yeasts but not of filamentous fungi (203) can be inactivated by various phosphatides, probably through the formation of lipoproteins which cannot penetrate the cell (204).

Metabolism of Microorganisms

Chemosynthesis.—The existence of organisms capable of oxidizing only inorganic substances, in accordance with Winogradsky's definition of "inorgoxidants," has become more and more doubtful. Iron bacteria such as *Leptothrix* species can grow in organic media devoid of ferrous ions (205 to 208), just as the typical sulfur bacterium *Beggiatoa* has been cultured in the absence of sulfide (209). Even

those autotrophic bacteria which fail to develop under such conditions carry out an oxidation of organic cell materials when the specific inorganic metabolites are not available. This has now been shown for nitrifying bacteria (210) and for *Thiobacillus thiooxidans* (211, 212). In the latter, R.Q. measurements indicate that the substrate is a carbohydrate. Determinations of bacterial polysaccharide fully substantiate this.

The oxidation of elementary sulfur by *Thiobac.* involves a preliminary dissolution of the substrate in fat globules located at the cell periphery (213, 214). During the oxidation, substances are produced with which carbon dioxide can be assimilated (215, 216). This was decisively demonstrated in experiments in which cell suspensions were allowed to oxidize sulfur in the absence of carbon dioxide. Upon cessation of this oxidation process due to exhaustion of the metabolite, the addition of carbon dioxide was followed by its rapid assimilation which is independent of the presence or absence of oxygen. The mechanism concerned in the transfer of energy from the oxidative to the assimilatory process appears to be a phosphate cycle with storage of the energy as phosphate bond energy and its release by subsequent splitting of phosphate esters (217). Thus the concept of the coupling of oxidative and assimilatory reactions by means of phosphorylations, so ably exposed by Kalckar (218) and Lipmann (219) for reactions not involving carbon dioxide ultization, has been broadened to include also the latter phase.

Gaffron discovered that green algae can develop by chemosynthesis, oxidizing molecular hydrogen in the dark, accompanied by carbon dioxide assimilation (220, 221). Under favorable conditions half as much carbon dioxide can be reduced as oxygen, which is a high efficiency for a chemosynthetic process. The implications for an interpretation of photosynthesis will not be discussed here.

General oxidation and reduction processes.—The new studies on the microbial oxidation of hydrocarbons (222 to 225), lower aliphatic alcohols, fatty acids, and simple benzene derivatives (226 to 229), chitin (230, 231), and lignin (232) add little to the understanding of these processes although they may aid in the differentiation of species. Rather unexpected is the reported ability of *Streptococcus* species to oxidize most of the lower alcohols (232a). More fully investigated from the biochemical point of view are the following: the oxidation of cholic acid by *Alcaligenes fecalis* (233), which seems to proceed by way of mono- and di- to triketocholanic acid; the decomposition of

amino-sugars (234) by yeast and bacteria with the production of ammonia and, at least in case of *Esch. coli* under anaerobic conditions, of acids; and the oxidation of amines by a number of common bacteria, especially *Ps. aeruginosa* which attacks most of the compounds tried (235, 236). Gale (236) has published quantitative measurements of oxygen consumption, carbon dioxide evolution, and ammonia production by washed suspensions which indicate a complete breakdown of the substrate to these end products. An inspection of Fig. 5 in his paper, a graphical presentation of the oxygen consumption with time, convinces one that the oxidation proceeds in steps which could be more closely analyzed if determinations of carbon dioxide and ammonia were not limited to the final stage. The resemblance between the oxidation of putrescine and agmatine, both involving the rapid uptake of 3 moles of oxygen followed by a slower consumption of 2.5 moles of oxygen per mole of substrate,[5] is striking, but the phenomena are not interpretable as long as it is unknown whether the ammonia is split off during the former or latter phase. The production of indole by *Esch. coli* is inhibited by the presence of glucose and phenylalanine (237, 238). While indole formation has usually been considered as the result of a decomposition of the tryptophane side chain, Krebs *et al.* (239) have suggested a mechanism whereby the primary attack occurs at carbon atom 2 of the indole ring, leading to an oxindole derivative, after which the ring is split to form an *o*-aniline compound. This attractive concept makes it possible to view the various types of tryptophane breakdown (with the formation of kynurenine, kynurenic acid, anthronilic acid, and indole) as modifications of one general scheme. It is supported by the observations that *Esch. coli* produces indole from *o*-amino-β-phenyl ethanol but fails to do so from indole derivatives other than tryptophane (240).

The linking of substrate oxidation to the reduction of components of the medium other than oxygen occurs in such processes as nitrate and sulfate reduction. The former is common among microorganisms, the latter restricted to a single type. *Proteus vulgaris*, for instance, can grow anaerobically in nitrate media with any substrate which can cause growth aerobically (241). The reduction of nitrate may lead to nitrite, nitrogen, or ammonia, depending upon the organism and the conditions (242, 243). Of special interest is the case of *Achromobacter arcticum* (243) which can reduce nitrate to nitrogen

[5] These are approximations; regrettably all data are "corrected" for oxygen consumption by blanks, and the extent of the correction is unknown.

but not to ammonia, and hence requires both nitrate and ammonia for anaerobic growth in simple media. The production of nitrogen, when nitrate or nitrite is added to sewage containing abundant carbohydrate, gives rise to nitrogen losses (244).

Sulfate reduction, also known to occur with a wide variety of simple organic substances and with molecular hydrogen, has now been announced to take place even in the absence of any reducing agent, accompanied by growth of the causal organism if an atmosphere containing 10 per cent carbon dioxide is supplied (245). Although from the published report a flaw in experimental technique is not apparent, the reviewer feels that it is preferable to conclude that such must have existed since the only alternative would be that our most elementary concepts of chemistry are fundamentally wrong.

Theoretically possible but experimentally much disputed is the occurrence of a phosphate reduction. The latest publication (246) contains valuable methodological details indicating that previous reports of a positive nature must be regarded sceptically.

The bacterial reduction of tetrathionate (247) resembles more closely the "phytochemical reductions" in which the reducible compound functions only occasionally, instead of generally, as hydrogen acceptor. Used for many years by Fischer (247a) for preparative purposes, the application of photochemical reductions to accomplish delicate reductions is spreading. In the steroid group the conversion of carbonyl to carbinol groups can be achieved with mixtures of yeast and sugar or with various bacteria (248 to 252); the reduction of tetrazolium to formazan compounds, taking place within the cells of many microorganisms (253), is biologically of importance because the strongly red-colored "leucoform" is stable in the presence of air, thus simplifying redox measurements in the range from -0.17 to -0.26 v. Just why the disappearance of double bonds in mineral media containing oleic or sorbic acids as the only carbon compounds is ascribed to a "hydrogenation" by the developing bacteria (254) is not clear; it seems far more likely that the acids are broken down by oxidation.

Polysaccharide metabolism.—There has long existed a controversy as to whether certain experimental results indicate a "direct attack" on di- and polysaccharides as contrasted with the more conventional concept of a previous hydrolysis. The main arguments in favor of the former view have been: (*a*) better growth with and faster decomposition of complex carbohydrates than of hexose sugars; (*b*) inability of certain microorganisms to attack the simple constitu-

ents of a utilizable polysaccharide; and (*c*) formation of different metabolic products from hexoses and from di- or polysaccharides.

Some recent experiments have corroborated the first point (*a*), but interpretations have again been opposite. Since *Fusarium lini* grows faster on trehalose than on glucose and ferments the former more rapidly (255), it was concluded that the disaccharide must be fermented "directly." A similar argument is based on the fact that while the *in vitro* activity of maltase is negligible at pH 4, maltose is fermented by yeast at a still lower pH (256). *Ps. saccharophila* also grows better on starch, maltose, or sucrose than on glucose or fructose (7), and similar observations have been made with plant root cultures (257). But Doudoroff reasoned that it is more probable that the polysaccharides are primarily split by a phosphorolysis comparable to that found by Cori for glycogen (258).

The second point, (*b*), has been observed with certain microorganisms causing the breakdown of agar and cellulose, but it has not been substantiated by recent work. The failure to detect carbohydrate decomposition by agar-decomposing bacteria must be ascribed to inadequate techniques used (259), while with cellulose-decomposing organisms (260) it appears due to a decomposition of glucose during sterilization by heat (261). Growth of the typical cellulose-decomposing *Cytophaga* species has been ascertained with glucose or cellobiose as only carbon sources when the sugars were sterilized by filtration (179).

To the third category, (*c*), belong the reported production of acid from sucrose and of alcohol and carbon dioxide from glucose by *Candida albicans* [see (262)], and the formation of polysaccharides like the levans and dextrans synthesized by lactic acid bacteria from sucrose but not from invert sugar (263, 264, 265). Experiments on the metabolism of *Cand. albicans* have shown that an intrinsic difference in the decomposition of sucrose and the hexoses does not exist (8). Polysaccharide formation may well constitute another example of a spontaneous enzymatic synthesis from special hexosephosphates, similar to the enzymatic synthesis of starch and glycogen from Cori-ester (266, 267). The enzymatic production of dextran with filtrates of *Leuconostoc mesenteroides* (268) makes it possible to test this hypothesis. The dextran of *Ps. tumefaciens* (269) may also belong in this group. The polysaccharides isolated from tubercle bacilli and *Eberthella* appear to differ considerably (270, 271).

Hence it becomes possible to reconcile the conflicting views on the

decomposition of polysaccharides by suggesting that in a number of cases these complexes are split by phosphorolysis instead of hydrolysis, thus avoiding such vague terms as the production of "activated" hexoses by the splitting or equally indefinite concepts of a "direct attack." Particularly the views on cellulose decomposition have become clarified: Boswell's attempts to explain the formation of bacterial gums by oxidation of lateral and terminal hydroxyl groups of the cellulose molecule (272), as well as the previous accounts of "oxycellulose" production so carefully analyzed and criticized by Norman & Fuller (273), are superfluous since the same substances are also produced from glucose and cellobiose (179).

The anaerobic decomposition of cellulose remains a confusing problem. Most experiments here seem to have been carried out with impure cultures which accounts for much of the variation observed (274, 275, 276). A critical and careful study of this fermentation is badly needed.

Renewed interest has been shown in the remarkable crystalline dextrins produced during the degradation of starch by *Bacillus macerans*. Excellent methods are now available for the preparation of enzymes, and a beginning has been made in the characterization of the products, now designated as cyclohexa- and cyclohepta-amylose to replace the older designation of α and β dextrins (277 to 281).

Oxidative sugar metabolism.—The idea of a close relationship between *Pseudomonas* and *Acetobacter* species, heretofore restricted to morphological considerations, has been strengthened by the discovery that the oxidation of sugars in high concentrations by the former results in high yields of products of incomplete oxidation like gluconic and ketogluconic acids (282, 283, 284), so characteristic of the action of the latter. In view of the simpler nutrient requirements of most *Pseudomonas* species this may be of some technical importance, and their ability to attack such a wide variety of substrates indicates potentialities for application. *Acetob. suboxydans* has been used successfully for converting α-glucoheptitol to *l*-glucoheptulose (285).

Lactic acid fermentation.—A method has been worked out for the preparation of active cell suspensions of lactic acid bacteria (286); with such material it has been shown that all streptococci convert sugar almost completely into lactic acid as they also do in growing cultures (287). During the fermentation a conversion of inorganic into organic phosphate occurs (288) in agreement with the prevalent theories on the mechanism of this process. A rather unexpected con-

tribution is the demonstration that in an alkaline environment homofermentative lactic acid bacteria produce considerable amounts of alcohol and volatile acids in a ratio of two formic to one acetic acid (289). It was also observed that at high pH and with high phosphate concentration much mucus is formed in glucose media.

Pyruvate fermentation by growing cultures of lactic acid bacteria does not follow the equation established by Krebs with cell suspensions. In addition to lactic and acetic acids, acetyl methyl carbinol and 2,3-butylene glycol were found (290). Data for carbon dioxide production are, unfortunately, missing, and since a calculation shows a lack of agreement in the composition of the products as compared with the substrate, it is possible that the figures for acetic acid may include formic acid which was not determined separately.

Lactic acid production from hydrolyzed Jerusalem artichokes has been carried out with aerated cultures of a sporeforming bacterium (291).

Sugar fermentations of the Coli group.—The *Proteus* species cause a sugar fermentation which closely resembles that of the coli-aerogenes group, *P. vulgaris* being similar to *Escherichia* while *P. mirabilis* and *P. hydrophilus* are more like *Aerobacter* (292, 293). The type of fermentation and the polar flagellation of *P. hydrophilus* suggest its identity with *Ps. fermentans* (294) or *Aerob. liquefaciens* (294a). Such fermentations have also been described for luminous bacteria (295) and may be far more general among polarly flagellated species than has hitherto been supposed. With *Photobacterium* two types of fermentation can be distinguished. Formic, acetic, lactic, and succinic acids are formed in both, along with ethyl alcohol. Members of the *Ph. phosphoreum* group liberate hydrogen, which is in increased amount upon the addition of formate, while hydrogen is not evolved by *Ph. fischeri.* The two fermentations are, therefore, identical with those of *Escherichia* and *Eberthella,* respectively.

Experiments with enzyme preparations of *Escherichia* and *Aerobacter* cells have established many of the step reactions in these fermentations such as: (*a*) the reversible splitting of hexosediphosphate to glyceraldehyde phosphate and dihydroxyacetone phosphate and the interconversion of the last two compounds (296); (*b*) the oxidation of phosphoglyceraldehyde to phosphoglyceric acid (297); (*c*) the reversible transformation of phosphoglyceric to phosphopyruvic acid and the phosphate transfer from the latter to adenylic acid (298); and (*d*) the reduction of acetaldehyde to ethyl alcohol (299). It may well

be concluded, as Utter & Werkman have done, that the Embden-Meyerhof scheme of glycolysis is fully applicable to these bacterial fermentations. In that case the glyoxalase found in *Esch. coli* (300) may not play a significant part.

Special investigations on the activities of separate enzymes on pyruvic acid have also been revealing. From *Esch. coli* a system has been separated (301) which oxidizes pyruvic acid in agreement with the equation:

$$CH_3COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2.$$

The absence of carbon dioxide production under anaerobic conditions eliminates a dismutation of pyruvic acid as a regular process while the fact that acetaldehyde is not appreciably oxidized shows that an ordinary decarboxylation of pyruvate is not responsible for the acetic acid production. The system which requires cocarboxylase, phosphate, and magnesium or manganese ions resembles the one found by Lipmann in lactic acid bacteria.

Contrasted with this is the enzyme from *Aerobacter* cells (302) which acts on pyruvate as follows,

$$2CH_3COCOOH \rightarrow 2CO_2 + CH_3COCHOHCH_3,$$

and which behaves in many respects like the carboxylase obtained from animal tissues (303, 304). Its active ingredients again comprise cocarboxylase and magnesium or manganese. Since the formation of acetylmethylcarbinol does not seem to require a special "carboligase" (305, 306, 307), it is probable that the main difference between the *Escherichia* and *Aerobacter* enzymes which act on pyruvic acid resides in the protein constituents. A discrepancy between the *Aerobacter* enzyme and that from animal sources should, however, be emphasized. The latter produces a higher yield of carbinol from pyruvate when acetaldehyde is added to the system and converts added propionaldehyde into methylpropionylcarbinol, whereas the former does not appear to couple free aldehyde with that released from pyruvate. Further experiments with various aldehydes are here in order, especially because in the sugar fermentation by *Aerob. polymyxa* (308) addition of acetaldehyde does increase the yield of acetylmethylcarbinol. As long as sugar is present the low redox potential causes a reduction of this substance to 2,3-butylene glycol.

As could have been expected, the presence in cider of acetylmethylcarbinol and butylene glycol results from the participation of *Aerobacter* species in the fermentation (309).

Decarboxylation of oxaloacetate by preparations of *Micrococcus lysodeikticus* (310) has furnished the first conclusive evidence for the existence of a β-decarboxylation. The enzyme concerned must be different from the above-mentioned carboxylase since with cocarboxylase-deficient cells, activation is possible by magnesium or manganese in the absence of cocarboxylase. Also, the "animal carboxylase" does not act on oxaloacetate (304). Reports on the decomposition of oxaloacetate by *Esch. coli* are conflicting. On the one hand, Deotto (311) claims that the substrate is attacked only aerobically and by S-strains, R-variants do not oxidize it, and neither type causes an anaerobic breakdown. Lehninger, on the other hand, has shown an anaerobic decomposition to 2 moles of acetic acid per mole of oxaloacetate (312). The latter results were obtained in an acid medium where a purely chemical decomposition was minimized. This may well explain the discrepancy.

Decomposition of citric acid.—The previously postulated mechanism[6] of citric acid fermentation *via* an initial breakdown to acetic and oxaloacetic acid has received support from studies with *Streptococcus paracitrovorus* (313, 314). Also, the aerobic experiments with *Aerob. indologenes* (315), showing that the first step consists of an attack which does not involve the participation of oxygen, are not inconsistent with that view. The oxidation is typically that of the fermentation products, acetic and succinic acids. The anaerobic decomposition by *Aerobacter* and *Citrobacter,* however, presents features not so easily accounted for by this mechanism because *Citrobacter* species do not form enough acetate and too much carbon dioxide to explain the high succinic acid yields in a simple manner (316). The fundamental differences in citrate fermentation by *Aerobacter* and *Citrobacter* are of value in differentiating the "citrate positive" strains of the coli-aerogenes group.

Sugar fermentation by anaerobic bacteria.—In the butanol fermentation hydrogen is not produced by a splitting of formic acid. Neither living cells, prepared by a special procedure (317) of which the chief feature may be the exclusion of oxygen, nor the enzyme preparations from frozen and thawed cell material decompose formic acid (318, 319). The enzyme acts on pyruvic acid as follows,

$$CH_3COCOOH \xrightarrow{(+H_2O)} CH_3COOH + CO_2 + H_2,$$

[6] *Ann. Rev. Biochem.,* **10**, 575 (1941)

but it is inert towards sugar and lactic, succinic, and 3-phosphoglyceric acids. Dilution inactivates it: activity can be restored by boiled cell juice or liver extract though not by cocarboxylase, flavoadenine nucleotide, coenzyme I, muscle adenylic acid, or divalent metals (319). The cell suspensions, which rapidly deteriorate unless stored in the presence of glucose at a temperature where fermentation is not stopped, break down pyruvate to acetic acid, apparently without hydrogen production. This suggests the transfer of hydrogen to some acceptor which, however, cannot be butyric acid since this is reduced only in the presence of glucose. Extra acetone is produced from added acetate provided pyruvate or glucose is present. Davies (318) suggests the formation of acetopyruvate from pyruvic and acetic acids with a subsequent decarboxylation to acetoacetic acid and to acetone. Yet acetopyruvic acid is not attacked. More likely, therefore, is the synthesis of acetoacetate from phosphopyruvate (or acetylphosphate) and acetate. Extracts from acetone-treated cells contain an enzyme which decarboxylates acetoacetic acid.

Technical details of butanol fermentations from agricultural products (320, 321) and of recovery of riboflavin from the fermented liquors (322, 323) are not discussed here.

Clostr. tetani does not ferment sugar; the positive results[7] reported previously were due to the use of impure cultures (324, 338). The fermentations by anaerobic sporeformers and streptococci described by Prévot *et al.* (325 to 328) are not just sugar decompositions—witness the formation of ammonia, sulfide, etc. The quantitative analysis by means of a Duclaux distillation (329) is inadequate to determine the relative amounts of fatty acids when more than two are present.

The sugar conversion by *Clostr. thermoaceticum* in which acetic acid is practically the only end product (330) is strikingly reminiscent of that by *Clostr. aceticum* (331). Since the latter produces acetic acid also from hydrogen and carbon dioxide, the formation of more than two moles of acetate from one mole of hexose does not rule out a fermentation mechanism with the common one- and two-carbon intermediates in one-to-one ratio. A comparable synthesis of carbon chains is that of caproic acid from ethanol (332).

Glucose increases ϑ-toxin production by *Clostr. welchii* at all pH values and shifts the most favorable pH from 7.5 to 8.0, probably through acid production (333). Also, α-toxin yields are affected; it

[7] *Ann. Rev. Biochem.*, **10**, 576 (1941)

is formed best at pH 6.0 without sugar, at 7.0 to 7.5 with sugar. The α-toxin has a specific enzymatic effect on lecithin, splitting it into phosphocholine and a diglyceride (334).

Amino acid decomposition.—Gale's review (335) is a good introduction. Different enzymes act upon histidine, tyrosine, and β-(3,4-dihydroxyphenyl) alanine (dopa), as shown by "competition experiments" (336) and by the limited number of different amino acids attacked by *Clostridium* and *Proteus* species (337). Quantitative data on amino acid decomposition by *Clostr. welchii* and *Clostr. tetani* (338, 339) are as yet too incomplete to permit an analysis of possible mechanisms. With neither organism has a "Strickland reaction" been observed, nor does either split formic acid, so that hydrogen production by these organisms again has to be ascribed to another mechanism.

Detailed accounts of the fermentation of uric acid and related purines[8] are now available (340, 341). Glycine appears to be a likely intermediate product. The highly specific nature of the bacteria renders them suitable for qualitative and quantitative determination of purines.

Hydrolysis of arginine by streptococci[9] has been observed for all species of serological groups A to G (342); the viridans group lacks this ability while in the lactic group it is characteristic for *Strep. lactis*. The absence of urease has been confirmed. The production of ammonia and carbon dioxide from peptone is thus probably due to the arginine content of peptone.

Bacillus subtilis forms an extracellular polypeptide in a medium containing glutamic acid, citric acid, and glycerol (343). The peptide contains only glutamic acid. Tracer studies have shown that the synthesis must proceed to at least 30 per cent without rupture of the carbon-nitrogen bond and that α-imino glutaric acid, or some derivative of it, is directly involved in peptide formation.

Enzyme production.—Examples of the effect of environmental factors on enzyme formation include the repression of synthesis of various deaminases by glucose (344). This inhibition is not due to the acids produced from sugar. An interesting teleological discussion of such phenomena (345) leads to the recognition of compensatory and detoxication mechanisms, in addition to adaptive enzyme formation of which galactozymase production appears rather general (346, 347).

[8] *Ann. Rev. Biochem.*, **10**, 577 (1941)

[9] *Ibid.*, **10**, 569 (1941)

In many cases adaptive enzyme formation may, however, be a misinterpretation, and the results should be explained as due to selective growth of mutants (348 to 351).

The cytochrome systems of *Esch. coli, Strep. pyogenes,* and type I pneumococci are different from those of the higher animals (352, 353) while the protozoan, *Tetrahymena geleii,* contains the normal complement (354). An artificially produced, cyanide-stable variant of *Sacch. cerevisiae* (355) displays neither cytochrome oxidase nor cytochrome-c activity. As would be expected, the fermentative metabolism remains unimpaired.

Assimilatory processes.—It is now well established that even "resting cells" convert only part of the substrates into oxidation or fermentation products while a large fraction appears as cell materials or reserve products (6, 7, 8, 121, 315, 356 to 359). Dinitrophenol, azide, etc., increase the oxygen consumption to the theoretical values for complete oxidation.[10] This has been interpreted as inhibition of assimilation. Recent experiments (360) have shown that this is not necessarily true. In some cases the addition of dinitrophenol, after the rapid initial phase of oxygen uptake has passed, is followed by a rise in the rate of respiration to the initial value which persists until the substrate oxidation has become complete. The inhibitors may, therefore, cause a rapid oxidation of assimilated materials.

The mechanism of such assimilations, sometimes regarded as a "Pasteur effect," is not yet understood (361). The present tendency is to ascribe it, linked with phosphorylation reactions, to a reversal of the normal path of carbohydrate breakdown (218, 219, 362). Though there is much in favor of this concept, crucial experiments are still lacking. An investigation similar to that of glycogen formation from labelled lactate (363) but with properly selected microorganisms instead of animal tissues would go far toward establishing the main features of assimilatory reactions. Complications arising from endogenous metabolism could thus be avoided. A variety of simple substrates could be used, including compounds with characteristic structural groups, so that the interrelations of substrate configuration and assimilation could be much more readily interpreted.

Permeability.—At last the mechanism of ion absorption as related to metabolism (364) begins to become clarified. It has now been demonstrated that the absorption of sugar by various cells is accom-

[10] *Ann. Rev. Biochem,* **7,** 503 (1938); **10,** 563 (1941)

panied by an equally rapid penetration of potassium (365 to 369). Since the substrate undergoes a primary phosphorylation, as shown by the disappearance of inorganic phosphate, and the phosphate cannot enter except as a salt (370), the mechanism no longer presents the baffling aspect of accumulation against a gradient; the internal concentration of inorganic phosphate must be negligible during active metabolism. In line with this is the observation that both phosphate and potassium are again excreted as the sugar decomposition proceeds. Contrary to the view of Leibowitz & Kupermintz (369), these phenomena do not argue in favor of an initial transformation of hexose into polysaccharide; the same effects should be observable with substrates other than sugar such as ethyl alcohol, for which a phosphate disappearance has been established with yeast (371) but where a preliminary conversion into glycogen is most improbable.

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THE ELECTRON MICROSCOPE IN BIOLOGY

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The review which follows is to some extent different from those usually presented in the *Annual Review of Biochemistry*. Being a first report in this publication on the electron microscope, its scope, naturally, is broader than that of a report on the latest achievements in this particular field. It has to include some of the background and part of the historical development to complete the picture of the present status. This review has also, more or less, the character of a celebration of the electron microscope in that its entire development can be compressed into the last ten years. Without being able to set a definite date as a birthday of the electron microscope, we can consider the building in 1932 of the first compound instruments as the starting point for electron microscopy.

Historical

Electron microscopy had its inception in the recognition of the optical properties of a beam of electrons and of the optical behavior of magnetic or electrical fields acting on such a beam. Ever since cathode rays have been known, optical terms have been used in connection with them. The use of such terms at the outset was done without much conviction, merely denoting some vague analogy observed by some workers in this field.

The fact that the behavior of an electron beam in a magnetic or electric field could be described in terms of geometrical optics was not recognized until 1926. At that date, Busch (1) calculated the trajectories of electrons in combined axially symmetrical magnetic and electric fields and came to the conclusion that such fields act on an electron beam the same way as a lens acts on a light beam. Moreover, he found that this behavior could be completely described by a well-known law of geometrical light optics, namely, that one could ascribe to such a field a focal distance, f, an object distance, u, and an image distance, v, and that these distances were connected by the relation:

$$1/u + 1/v = 1/f \qquad \text{(I)}$$

In spite of the recognition of this optical behavior, five more years elapsed before mental inertia could be overcome to consider the pos-

sibility of using electrons for forming images. About 1931, however, workers in various laboratories became aware of the fact that electrons could be used for such purposes and that the relation expressed by equation (I) was more than a formal analogy. One of the first who seems to have recognized these possibilities was Rüdenberg (2) who developed the complete idea of an electron microscope in a German patent application, dated May, 1931. His patent, however, influenced the development of electron optics very little because it was not followed by any experimental work on his part. Much more important was the work done at the same time by Davisson & Calbick (3) in the United States, who not only experimentally demonstrated the formation of an electron image by means of an electrostatic field, but also calculated the optical constants of such a "lens." A little later Knoll (4, 5) and his students, Ruska and von Borries in Germany, took up the subject and built a magnetic instrument for the observation of electron-emitting surfaces, whereas the electrostatic counterpart of such an instrument was developed by Brüche (6, 7) and his collaborators, also in Germany. A magnetic instrument built by Marton was in operation in Brussels before the end of 1932.

Primarily designed for the observation of electron-emitting surfaces, all these instruments were used for a number of industrial problems, mostly connected with the operation of oxide-coated cathodes. Although some observations in transmission were made, it was not until 1934 that trend toward systematic application of a transmission type microscope became evident. By this time, two compound instruments were built for transmission observations alone, one by Ruska (8) and one by Marton (9). The latter instrument was used for the first biological applications of the electron microscope (10). The results were encouraging enough to warrant construction of a more elaborate instrument, the description of which was first given in December, 1934 (11). It embodies such factors as air locks for changing specimens and photographic plates without impairing the vacuum, a mechanical stage of the cross movement type, and an electrostatic shutter system for the control of exposure times (12). Independently of Marton, proposals for the inclusion of such elements were published in 1935 by von Borries & Ruska (13). In 1937 the description of an instrument constructed by Martin, Whelpton & Parnum was given (14). Early in 1938 von Borries & Ruska (15) published the description of the instrument which became the prototype of the commercially produced electron microscope of the Siemens factories (16).

Description of further instruments followed more or less rapidly. In 1939 Burton, Hillier & Prebus (17, 18) of Toronto constructed a magnetic transmission type instrument. The same year saw a description of an electrostatic instrument of high resolution by Mahl (19, 20). In 1940 came the description of two further magnetic instruments, one by von Ardenne (21, 22) and one by Marton (23, 24), the latter being the first instrument built in the laboratories of the Radio Corporation of America. The commercially produced RCA instruments were described by Zworykin, Hillier & Vance (25). Further technical details are contained in patents by Marton (26).

A description by Prebus of another magnetic instrument appeared in 1942 (27). A simplified electrostatic instrument was recently announced by Bachman & Ramo (28), and a reduced size magnetic instrument by Zworykin & Hillier (29). A number of other instruments are either in operation or construction. Those which deserve special mention are at the Massachusetts Institute of Technology, at the California Institute of Technology, at Eastman Kodak Co., and at Stanford University.

Resolving Power

The great importance of the electron microscope is in the increased resolving power. From the early days it has been suspected (4, 30) that the Abbe formula expressing the resolving power, d, of an optical instrument as a function of the wave length, λ, and the numerical aperture, $2n \sin \alpha$, as follows:

$$d = \lambda/2n \sin \alpha \qquad \text{(II)}$$

can be applied to electron optical systems, whereby α is the solid angle under which the lens sees the object and the refractive index has to be replaced by an equivalent expression. By 1935, mathematical proof was given for the validity of this assumption, first by Henneberg (31) and later by Henriot (32). By assuming for the electron the wave length derived from de Broglie's theory, the resulting theoretical resolving power of the electron microscope seems to carry us into atomic dimensions. By 1938, however, it was clearly recognized that aberrations of the electron optical elements do not permit the use of very high numerical apertures (33). By taking into account these aberrations and the resulting limitation of the numerical aperture, a theoretical limit of about 10 Å units was calculated for the existing electron optical systems (34).

The best observed value up to date is 22 Å units (22). In comparison, if the useful magnification of a light microscope with a resolving power of 0.2 μ is 2000 diameters, 22 Å resolving power of the electron microscope corresponds to a useful magnification of 180,000 diameters and 10 Å to 400,000. The resolving power of 0.1 μ of the ultraviolet microscope corresponds on the same scale to a useful magnification of 4000 diameters.

The Mechanism of Image Formation

In a transmission type of electron microscope the image is not formed by absorption or by refraction of rays in the substance of the specimen but by scattering. The incoming electrons are deflected on reaching the molecular or atomic fields of individual molecules or atoms of the specimen. As long as the thickness of the specimen is small enough in comparison with the mean free path of the electron and the chances for a second deflection during the passage of the beam are low, we speak of single scattering. When the thickness of the specimen is such that the probability of the individual deflection for a single electron during passage through the specimen is great, the phenomenon is known under the name of multiple scattering. Whereas the laws governing the scattering of electrons in a single process are more or less known and those for the multiple scattering can be statistically calculated, there is a range in between, known as plural scattering, which defies calculation. This range comprises a limited number of individual deflections (the number being from two to five) for which the laws of single scattering become too complicated and the statistical laws cannot yet be applied. Calculations of the intensity of the transmitted electron beam through the specimen were attempted at an early date by Marton (35) and later by von Ardenne (36). The most complete results up to now are contained in a recent paper by Marton & Schiff (37). It can be summarized as follows: If i_o is the intensity of the incident beam, i the intensity of the transmitted beam, x the thickness of the specimen, and N the number of scattering atoms per cc., the relation between these quantities can be given as:

$$x = (1/SN) \log_e (i_o/i). \qquad \text{(III)}$$

The expression S is the total cross section of scattering atoms which can be calculated as the sum of three kinds of scatterings: (*a*)

Elastic scattering, whereby the electron is deflected without loss of energy; (*b*) inelastic scattering combined with energy loss; and (*c*) the so-called free electron scattering. This latter, however, has to be considered only in case of metals, and, therefore, we can neglect it completely when dealing with biological specimens. As the number of atoms per cc., N, are equal to $N_o\varrho/M$, where ϱ is the density of the substance, M the atomic weight, and N_o the Avogadro number, the intensity of the transmitted beam is roughly proportional to the thickness and to the density of the specimen and inversely proportional to the electron speed as expressed in terms of the accelerating potential. If the intensity ratio is measured either by photography or by any other means, such a relationship as (III) can be used for the calculation of the object thickness. It has been found that for crystalline substances the relationship cannot be applied with any great accuracy due to internal Bragg reflections on the lattice planes (38). Most biological material, however, can be considered amorphous, and the thickness thereof can be determined with reasonable accuracy if the density is known.

Stereoscopic observations.—Whereas the determination of thickness by intensity measurement and scattering ratio can be applied only for very thin specimens (in the region of single scattering), stereoscopic observation offers the possibility for thickness determinations of relatively thick specimens. The ranges of the two methods of observation can be made overlapping to some extent when using relatively great viewing angles for stereoscopy.

Following an early proposal by Ruska (39), von Ardenne was first to publish the description of a stereoscopic attachment for the electron microscope (21). The usual specimen holder was replaced by one which could be tilted by several degrees toward the optical axis, and successful pictures of the same specimen could be taken at different degrees of tilt. Such a procedure, which cannot be applied at all in light microscopy due to the very limited depth of field of light microscopes, is very successful in electron microscopy. Because of the very limited numerical aperture of electron optical lenses, the depth of field is about 500 to 1000 times greater than for light objectives in spite of the increased resolving power. Parts of the specimen, therefore, which are several microns apart in depth, can be focused simultaneously on the same picture. Once such pairs of pictures are taken, they can be used for the determination of depth or thickness by the usual methods of stereophotogrammetry (35). The smallest thick-

ness measured by this method is about 200 Å units with an accuracy of ±50 Å units.

Another method for obtaining stereoscopic micrograms has been proposed by Marton. His pictures were taken by keeping the object stationary and changing the angle of incidence of the condenser beam (37). This method is applicable when the objective is used with relatively large apertures. Such large apertures can be used whenever very thin specimens are dealt with because the image quality is primarily defined for such thin objects by the solid angle of the incident (condenser) beam and not by the physical size of the objective aperture.

Penetration of the beam.—Up to the present time we have considered the electron beam corresponding to velocities between 50,000 and 100,000 electron volts (e-volts). The penetration of electrons of such velocity is rather limited and the upper limit in thickness for successful observation is below 1 μ. The range of penetration increases approximately proportionally to the square of the potential applied for accelerating the electron (41). Following a proposal by Marton (42), several instruments were built for use with higher voltages ranging from 200 to 300 kv., the first by von Ardenne (43), a second by Mueller & Ruska (44), and recently a third by Zworykin, Hillier & Vance (45). In spite of the scarcity of available publications, the results seem to be promising. The necessity for the use of even higher voltages is definitely proved in spite of the fact that no further increase in the resolving power can be expected from an increase in electron speed. The main reason for applying such high voltages is, besides the high penetration, the reduction of the energy losses in the specimen. Such energy losses affect the operation in two ways, by heating the specimen and by changing the image quality. The first effect gives a clue to a possible observation of living specimens: by increasing the electron speed, the heating is reduced and the chances of keeping the specimen alive increased. The description of a device for observing living matter with a 200 kv. electron microscope, reported by von Ardenne (43), has been followed by a second paper (available in abstract only) (46) on electron micrograms of spores of *Bacillus vulgatus* without impairing their germinability. The dosage necessary to kill the spore with the voltage used (180 kv.) is of the order of 10^5 electrons per spore.

The second point in favor of higher voltages is the reduction of chromatic aberration. Due to energy losses the speed of the trans-

mitted beam partly changes, and, therefore, it can no longer be considered a "monochromatic" beam after passing through a thick specimen. The chromatic aberration decreases with increasing electron speed.

Observation of the specimens at very high voltages becomes difficult due to the reduced contrast. The contrast can be calculated from scattering data and it would seem to become negligible for very high speeds. Fortunately, the theory does not seem to be quite satisfactory on this point; at least, observations of Mueller & Ruska (44) indicate that the contrast does not decrease as rapidly as claimed by the theory. It is particularly interesting in this respect, to refer to Fig. 9 in the publication of Mueller & Ruska (44), reproduced also by Anderson (47).

It might be interesting to point out that observation of contrast depends to a great extent on the degree of blackening of the photographic plate. There is an optimum for observation of contrast for blackening of the order of 0.8 (48).[1]

Technique of Observation

Whereas the very first microscopes operated with external photography (9, 41, 50) of the fluorescent screen, the use of internal photography has been very widely adopted ever since it was introduced in 1935 (12).[2] No marked changes have been described in this technique except for recently announced small size microscopes which show a tendency to go back again to the external photography (28). Such a procedure is quite justified when the microscope is not used to the limit of its resolving power. It is safe to presume that internal photography will remain the standard procedure for the highest magnifications while the external photography might be adopted for routine inspection work.

The proper magnification to be used in the electron microscope depends upon the choice of the photographic material. It has been shown by von Ardenne that having a given resolving power of the instrument, there exists an optimum magnification (51). This mag-

[1] Blackening is defined as the common logarithm of the ratio of incident to transmitted light.

[2] External photography is a picture taken by means of a conventional photographic camera of the image produced on a fluorescent screen, whereas in internal photography the photographic emulsion is exposed in the vacuum of the microscope to the electron beam.

nification is mostly of the order of 10,000 diameters. When using lower magnifications not all the resolving power of the microscope is used due to graininess of the emulsion and to scattering of the electrons in the thickness of the emulsion. On the other hand, the use of higher magnifications in the microscope itself is generally not worth while because all the details are contained already at the magnification of about 10,000 diameters, and higher magnification only reduces the size of the observed field. Standard practice is, therefore, to use relatively low magnifications in the microscope and enlarge the negatives to much higher magnification by photographic means.

Some of the more modern microscopes have been provided with means for observing the fluorescent image (either the main screen or intermediate screen) through magnifiers (16, 21, 23). These magnifiers can be mounted inside or outside of the vacuum. A different method of observation has been adopted lately by Marton (52) by omitting completely the use of intermediate screens and projecting an image of low magnification on the main fluorescent screen for orientation purposes. This can be achieved by means of a lens combination permitting a very wide angle of observation at a low magnification.

Focusing is generally done by viewing the image on the main fluorescent screen and by changing the strength of the objective lens. When dealing with virus particles or similar specimens of very small size and relatively low density, it is recommended generally to use some particles of higher density and greater size for focusing (53, 54, 55).

Technique of Preparing Specimens

Obviously there is no such thing as a standard technique for preparing biological specimens. Due to the wide variety of biological materials and the specific problems connected with each of them, each worker in the field has to devise his own method of mounting the specimens. The following, therefore, will be a brief survey of some of the applied techniques.

The conditions of image formation (37) indicate already that no standard glass slides can be used in electron microscopy because of their density and thickness. Also the use of cover glass is out of question because of the added scattering of the electrons. The standard procedure for mounting specimens is still the same as that introduced by Marton (56), that is, the use of very thin films of collodion or other plastics on which the specimen can be mounted. The average

thickness of the collodion films useful for such purposes is about 100 Å units. Directions for preparing such films are contained in papers by Ruska (57) and by Marton (58). The small apertures originally used for holding such thin films have been generally replaced, in the United States at least, by fine wire screens, varying from 100 to 400 meshes per inch (23, 58).

Bacteria and such specimens which can be suspended in distilled water are prepared by placing a small droplet of the suspension on the collodion film (56). The droplet is allowed to dry and the specimens are ready for observation. This technique was developed because of the difficulties of observation in saline or nutrient-containing solutions (58). In some cases, special techniques have been devised for observation of specimens which could not be transferred to distilled water. For instance, the so-called "wash-off" technique has been described by Mudd & Anderson (59).

> The minute drop containing the specimens is placed on the mount and the latter is held in a forceps for about thirty seconds so as to allow some of the bacteria (or other specimens) to settle on and adhere to the collodion film. The edge of the droplet on the mount is then touched to the meniscus of distilled water in a test tube held nearly horizontally. The droplet is violently propelled off the mount into the water by surface tension forces, leaving the sedimented bacteria on the collodion film relatively free of impurities. After this washing process has been repeated, the preparation is allowed to dry in air and introduced into the microscope.

Similar results have been achieved when allowing the bacteria to settle and the supernatant liquid taken off by means of either a micropipette or any other means permitting removal without violent motion of the liquid. For the virus particles, which cannot be suspended in distilled water, similar techniques are applicable.

The observation of histological specimens being still in its infancy, the reader is referred to very early publications by Marton (60) and by Krause (61), describing methods which are probably to some extent obsolete by this time.

Special techniques have been described for the observation of insect cuticle and insect tracheae by Richards & Anderson (62, 63), whereby freehand sectioning methods are used for cutting the cuticle which was inserted in a slit made in a turgid carrot. The tracheae were simply dissected and torn open for observation. A sectioning technique using a water-soluble wax as embedding medium was described by the same authors (64). A wedge sectioning method has been developed by von Ardenne (65).

For observation of nontransparent specimens, replica techniques have been introduced into electron microscopy. Such techniques consist in casting a very thin film on the surface of the specimen and after solidifying, peeling it off (66, 67). These techniques up to now have not been much applied for biological specimens.

Auxiliary Equipment

Besides the means for stereoscopic observation mentioned above, the most important auxiliary equipment of the electron microscope provides means for electron diffraction observation. First attempts by Martin, Whelpton & Parnum (14) were followed by practical applications by Ruska (68), von Ardenne (69), and by Hillier, Baker & Zworykin (70), which show definite promise. In a new construction, Marton (52) combines the advantages of previous designs without any intermediate manipulation of the specimen.

Freezing attachments have been built by Krause (61) and by Marton (23). Desirable as it might appear, no practical use for such equipment has yet been described. In this reviewer's opinion, however, a good freezing attachment might become a very precious auxiliary of biological research.

Microreaction chambers for carrying out small-scale chemical reactions in the electron microscope were described by von Ardenne (22, 71). No one seems to have attempted, up to date, the construction of a micromanipulator for use with the electron microscope.

Biological Applications

The applications of the electron microscope to biology may be roughly classified into the following groups: (*a*) morphology of bacteria; (*b*) virus research; (*c*) adsorption phenomena; (*d*) observations of induced changes; and (*e*) miscellaneous, including histology.

While these classifications may seem quite arbitrary, they fit reasonably well into the present status of electron microscopy. Each group will be treated by giving one or more papers, representative of the group, in greater detail and by enumerating other publications in the same field.

Morphology of bacteria.—The first observation of bacteria by means of the electron microscope was published in 1937 by Marton (56). After an attempt by Krause later in the same year, published in a much discussed paper (61, 72, 73, 74), von Borries & Ruska published in 1938 a considerable number of pictures of bacteria (75).

From then on, quite a number of papers have been published among which particular mention is made of the following: von Borries, Ruska & Ruska (76), Piekarski & Ruska (77), Piekarski & Ruska (78), von Ardenne (79), Burton, Hillier & Prebus (80), Frühbrodt & Ruska (81), Jakob & Mahl (82), Lembke & Ruska (83), Mudd & Lackman (84), Mudd, Polevitzky, Anderson & Chambers (85), Mudd, Polevitzky, Anderson & Kast (86), Mudd, Polevitzky & Anderson (87), Morton & Anderson (88), Umbreit & Anderson (89).

Particularly representative of this group is the paper by Lembke & Ruska and the series of four papers by Mudd and his collaborators. Lembke & Ruska (83) undertook to make comparative observations of the infective agent of tuberculosis using both the light and electron microscopes. Their observations were carried out on *Mycobacterium tuberculosis,* var. *hominis,* var. *bovis,* and *Mycobacterium avium.* They observed in all three types the presence of granules of varying sizes. The number of relatively large granules did not seem to exceed eight per individual cell, whereas the small elements, called for their purposes microgranules, were present in considerable quantity. Granules have average dimensions of 3000 to 3500 Å units, whereas the microgranules average 200 to 500 Å units in size. These measurements were all taken from the *Mycobacterium avium* organism. Besides granules and microgranules, they also observed brighter and therefore less dense areas which they called "vacuoles." How much of this structure is due to artifacts cannot be distinguished. However, it is stated that under an intensive electron bombardment of long duration the granules will partly become brighter and will finally be destroyed with a kind of explosion. The micrographs of cells treated with ethyl ether show a structural differentiation of the granules, leading the authors to the hypothesis that the granules might be built up of microgranules. Hydrolysis with 0.1 *N* hydrochloric acid does not change the appearance of the protoplasm. Likewise, the appearance of the granules remains intact.

No evidence was found for the presence of an external wax envelope. Very fine discontinuous layers can be seen on some cells. Evidence in the cited paper and in the micrographs taken in this country from the same subjects gives no indication of the existence of envelopes.

From all the available pictures, one gathers the impression that there are layers of absorbable material around the cell due to the process of preparing the specimen. However, as the authors point

out, there is no definite evidence against the presence of extremely thin layers of lipids which could not be more than a few molecules thick.

In a series of four papers, Mudd and his collaborators present evidence that the cell wall of the bacterium is actually a rather rigid membrane. The first of these papers (84) deals with structural differentiation within the streptococcus cell. They state that

> the outer surfaces of the streptococcal cells of a chain of bacteria have continuity throughout the chain. Adjacent cells may be separated only by a slight constriction, by a deeper constriction, by a relatively broad band or by a filament which is just resolved

by the instrument. Profile pictures of bacterium cells on broken collodion films show that "the external structure of the bacteria, at least, is maintained without collapse from desiccation or action by the electron beam."[3] Specimens subjected to short periods of sonic vibration show "ghost" cells from which the protoplasm escaped, but left the cell walls intact. Some micrographs showing the gray cell outlines with black axial portions are to be construed as indications of plasmolysis or shrinkage of the central protoplasm from the cell wall.

In the second paper (85), the results of investigations on the cell wall of *Bacillus subtilis, Bacillus megatherium,* and *Bacillus anthracis* are reported. Some of the specimens were subjected to sonic vibrations and the results observed in the electron microscope. Besides "ghost" cells, jagged edges of broken cells are observed and interpreted as further evidence for the rigidity of the cell walls. It is observed, particularly in the case of *Bacillus subtilis,* that spores seem to have greater density than that of vegetative cells. Observations of a great number of cells in which the protoplasm has shrunk away from the cell wall in drying induce the authors to criticize a paper by Jakob & Mahl (82), containing numerous electron micrographs of nonencapsulated anaerobic bacillae in which similar structures were interpreted as capsules.

The third paper (86) deals with the "Cell wall and protoplasm in a strain of Fusobacterium." Whereas previous papers contain observations of relatively opaque cells without any evidence for internal structure, fusobacteria give well-defined differentiation within the

[3] Evidence for such rigidity of *Hemophilus pertussis* cells was previously published by Marton (58).

protoplasm. Evidence is given for the existence of a cell wall as in previous cases.

The last paper (89) in the same series has as subtitle: "Structural differentiation within the bacterial protoplasm." The observations on *Mycobacterium tuberculosis* var. *hominis* are in agreement with the paper by Lembke & Ruska (83). *Corynebacteria, Vibrio,* and *Spirocheta* all show granules similar in character to the previously mentioned ones, ranging in diameter from 700 to 2300 Å units. No granules but dense areas of markedly different size, shape, and position, without being sharply circumscribed, are shown in cells of *Eberthella typhosa* and *Fusobacterium.* Micrographs show the existence of flagellae on *Spirocheta pallida.*

As a rule, most papers dealing with bacterial morphology are restricted to recording observed shape and size without attempting much of an interpretation. Workers in this particular field seem to feel that it is still too early to attempt to characterize their findings. They try to observe a very great number of bacterial cells under varying conditions and work out some standard method of observation.

Virus research.—It might be more justifiable to call this chapter morphology of virus particles. The publications in this group contain mostly pictures of different plant viruses: Kausche, Pfankuch & Ruska (90), Kausche & Ruska (91), Trurnit & Friedrich-Freksa (92), Kausche (93), Melcher, Schramm, Trurnit & Friedrich-Freksa (94), Stanley & Anderson (95), Holmes (96), and Frampton (97). A minority of the papers which deal with animal viruses are the following: Ruska, von Borries & Ruska (98), von Ardenne & Pyl (99), Kausche & Pfankuch (100), Ruska (101), Luria & Anderson (102), Anderson, Chambers & Henle (103). As a rule the electron micrographs confirm previous findings by indirect means about the shape and size of virus particles except in the case of bacteriophage, which should be discussed later in greater detail.

Several papers, both in this country and in Germany, are devoted to the subject of the morphology of tobacco mosaic virus. There is agreement with previous findings that the tobacco mosaic virus is a rod-shaped particle though there is still discussion about the exact size of the particles. Kausche, Pfankuch & Ruska (90) in a first paper report 150 Å for the width and 3000 Å for the length. However, they report the presence of considerable numbers of particles of only half this length. In a later paper, Melcher, Schramm, Trurnit & Friedrich-Freksa (94) find particles whose lengths were predomi-

nantly in the neighborhood of 1400 and 1900 Å. Stanley & Anderson (95) find basic lengths of 2800 Å for the length of the particle with about 20 to 30 per cent of the particles being of considerably shorter length. Frampton (97) criticizes these papers, and from his measurements of the particles on published micrographs, he comes to the conclusion that all lengths are multiples of a basic length of about 370 Å. Although Frampton's method of measuring typographical reproductions instead of the original micrographs is subject to criticism, it should be pointed out that there is no unanimity as yet about the size of the tobacco mosaic virus particle. Some of the uncertainty can doubtless be explained by differences in experimental technique, by aggregation, and the degree of purification of the specimens. This reviewer feels that more research work is indicated to explain the discrepancies between the published findings.

Bacteriophage anti-coli was first investigated by Kausche & Pfankuch (100) by means of the electron microscope. Their observations indicated that the phage has a tendency to penetrate into the attacked cell. Simultaneously, Ruska (101) gives evidence for "sperm-shaped" particles from a phage suspension. However, he was unable to decide whether such particles represented phage itself or other bacterial products. A recent paper by Luria & Anderson (102) comes to the following conclusion: Phage particles

> consist of a round "head" and a much thinner "tail" which gives them a peculiar sperm-like appearance. The "head" is not homogenous but shows an internal structure consisting of a pattern of granules. . . . . The diameter of the "head" appears to be about 800 Å units, the "tail" is about 1300 Å long. . . . . They appear to stick to the bacteria either by the head or by the tail. . . . . The number of particles adsorbed on a bacterium increases with the time of contact.

It is very interesting to note that the size of the "head" as shown by the electron microscope is in fair agreement with the figures deduced from the inactivation of the phage by x-rays as carried out by Luria & Exner (104), thus giving substantial evidence for the validity of the so-called "hit" theory for the determination of the "sensitive volume." As Luria & Anderson (102) point out, "this conclusion, too, seems to be interesting from the point of view of genetics, since the 'hit' theory, although widely criticized, has been used for calculating the approximate value of the dimensions of genes."

Special mention should be made of observations on influenza virus by Anderson, Chambers & Henle (103). Where earlier work indicated roughly 1200 Å units for the particle size, research carried out

by them indicates a particle of roughly 100 to 120 Å size to be the infective agent.

Adsorption phenomena.—A few papers have been published on observations of adsorption phenomena. They deal with adsorption on virus particles (53, 54, 105) and on adsorption on bacterial flagellae (106). As representative of this group of papers, mention might be made of the investigations of Kausche on the mechanism of the gold-sol-reaction on tobacco mosaic and latent mosaic viruses (potato-*X* virus) (105). The first interesting feature of this particular investigation is that both constituents of the adsorption reaction are within the range of visibility of the electron microscope. Also, both constituents are so stable that no induced changes are to be expected either by the technique of preparing the specimen or by the technique of taking the electron micrograph. The results can be summarized as follows:

The flocculation-reaction of gold with tobacco mosaic and potato-X virus proteins depends upon both the pH of the medium and of the surface of the protein. Colloidal gold is bound to tobacco mosaic only in strong acid reaction. The flocculation-reaction is essentially independent of the particle size of the colloidal gold used for the reaction.

Both colloids being negatively charged, no reaction can be expected between them. However, after adding sodium chloride to the potato-*X* or hydrochloric acid to any of the virus solutions, the virus particles become positively charged, and in a limited pH range a definite adsorption reaction can be observed. This rule is valid for the smallest colloidal gold particles observed of about 30 to 50 Å in diameter. However, the author is unable to tell what the coverage of the virus particle is. In this respect, this paper can be well compared to similar findings by Anderson & Stanley (54) on antigen-antibody reaction of tobacco mosaic virus. They are equally unable to tell whether or not the coverage is complete. Whether this has anything to do with the unchanged infectivity of the virus proteins after the gold-sol-reaction remains to be solved.

Observation of induced changes.—One of the most promising applications of the electron microscope to the study of biological objects is the possibility of studying induced changes on a scale which was completely unavailable before the advent of this new instrument. Such studies are still at their beginnings, but due to their importance, it might be worth while to point them out at some length.

Of course, the investigations of the action of bacteriophage on bacteria, mentioned in a previous section, should be included under the heading of induced changes. Observations of Kausche & Pfankuch (100) and Luria & Anderson (102) indicate that the bacterial cell, after contact with a phage suspension, shows striking changes in its appearance. Not only are the phage particles sticking to the attacked cell, showing something which might be interpreted as penetration of the cell membrane, but the cytoplasm, which had previously a uniform appearance in the case of *Escherichia coli,* now appears to be full of "holes."

Induced changes, however, are generally understood to be the changes caused by chemical or physical agents, and as such the action of germicides or of fixing agents would seem to be the most interesting subject for immediate observation. Characteristic of these investigations on induced changes is the paper by Mudd & Anderson (59). As outlined in the section on mechanism of image formation, contrast of the image is merely due to scattering of electrons. This scattering is the function of both the thickness and the density of the specimen. The observers' way of modifying the contrast is to attempt to change locally the density (thickness being a more or less constant factor). The first indications of the deliberate use of staining action by heavy metals was given by Marton (35). Mudd & Anderson (59) add silver nitrate, mercury bichloride, or lead acetate solutions at varied concentrations to bacterial suspension of *Fusobacterium,* of *Eberthella typhosa,* of *Shigella dysenteriae,* and of *Vibrio comma. Fusobacterium,* mixed with 3 *M* silver nitrate solution, shows cells with unstained walls but the inner protoplasm is rendered opaque. The use of 0.22 *M* mercury bichloride darkens the protoplasm and shrinks it away from the cell wall. By adding 0.59 *M* lead acetate, an even stronger coagulation and shrinkage of the protoplasm than with silver nitrate and mercury salts is noticeable. The flagellae of *E. typhosa* are destroyed by 3 *M* silver nitrate, whereas the action of lead acetate makes the flagellae appear darker without any apparent thickening. Lead acetate also swells the cells of *E. typhosa* and induces the protoplasm to escape from the cell.

Shrinkage of the protoplasm is observed on *Shigella dysenteriae* through the action of 0.14 *M*. solutions of silver nitrate, nickel nitrate, and lead acetate. Some staining of the protoplasm and exudation of the protoplasm as a result of the injury to the cell can be observed in the case of *Vibrio comma* treated with 0.29 *M* lead acetate. The

results can be summarized in that by use of heavy metal salts the cell membrane in most cases does not seem to be attacked. The salts are readily diffused through the wall and the stain will attack the protoplasm.

Another interesting case of induced changes is the observation by Morton & Anderson of *Corynebacterium dyphtheria* in a tellurite-containing medium (107). The bacterial cells show needle-like crystals which cannot be observed in any normal cell. These crystals disappear upon the addition of an oxidizing agent such as a bromine. The authors infer from this that the crystals are metallic tellurium and that the tellurite ion contained in the nutrient is able to diffuse through the cell wall and is reduced with the precipitation of tellurium metal within the cell boundaries. The apparent distortion of the cell wall by the needle-shaped crystals (not as apparent on the reproduction, but clearly visible on the original pictures) is taken as proof for the occurrence of the crystals inside the cell membrane. It might be worth while, however, to substantiate this latter claim by observation of the same crystals within the cells by means of stereoscopic micrographs.

Miscellaneous, including histological.—From a great number of various biological applications which cannot be clearly classified under the previous headings, a few should be mentioned in particular. The first paper of this group that should be included in this review is by Wolpers & Ruska (108) and deals with the coagulation of blood. To a large extent the investigation confirms earlier results obtained by indirect methods. In particular, they conclude that sufficient proof is given for the existence of micelles in blood fibrin. By comparing their observations in the electron microscope with dark-field observations on the light microscope, the authors form a definite theory of the mechanism of the coagulation processes, full details of which may be found in the original paper.

Observations by E. von Haam on thrombocytes of various species are reported in a paper by Prebus (27). It is to be hoped that this interesting research will be published in greater detail.

A very interesting field of research has been attacked in a recent paper by Schmitt, Hall & Jakus (110). The authors investigate the structural properties and mode of formation of collagen fibrils. Collagen has been the object of x-ray diffraction research for quite some time, and the review of the literature of the subject up to 1940 may be found in a paper by Astbury (111). A number of different

collagen fibrils have been prepared from seven different tissues: tail tendon, *tendo Achilles*, dermis of skin, interfibrillar connective tissue of nerve, cornea, spleen and *ligamentum nuchae*. These tissues were obtained from five species of mammals (white rat, beef, lamb, kangaroo, and human), one amphibian (frog) and one mollusk (squid).

From the different methods of preparing the fibrils, the one used for rat tail tendon might be typical. These were precipitated from the acetic acid solution by neutralizing with 0.1 *N* ammonia. Electron micrographs of such collagen fibrils show characteristic cross-striated appearance due to the presence of bands which are alternately of relatively high and low density.

The spacing which is defined as the distance along the fibril between bands of like density vary considerably from one fibril to another. However, a distribution curve made from measurements of about 200 fibrils shows that the most frequently occurring spacings lie between 620 and 660 Å units, the average value being 644 Å units. This coincides with the X-ray fibre axis long spacings of 640 Å reported by Bear (112), indicating that the structure revealed in the electron micrographs is responsible for the long spacing diffraction.

It is interesting to remark that Scott & Anderson (113) find striations on connective tissue from omentum and nerve sheaths and on subcutaneous connective tissue, muscle connective tissue, and perineurium with spacings of fibrils of 695 Å units. Besides the spacing of the striations, the authors mention the length of dark and light bands. Band length of about 440 Å for the bands of high density and 200 Å for bands of low density have been observed. The ratio of the length of these bands seems to be fairly constant and equal to about 2.2.

Further observations were made about the extensibility of collagen fibrils. Occasionally the collodion film on which the fibrils are supported is ruptured during electron microscopical observation due to electrostatic charges produced by the electron beam. When this occurs, the film bends back to the supporting wire and some fibrils are left suspended on the wire under considerable tension. In a few cases the entire process was observed visually and photographs were made of the stretched fibrils. The most striking manifestation of stretching is the relatively enormous increase in the spacing, which could extend to about 6000 Å units. In the opinion of the authors,

this indicates that isolated fibrils in distinction to intact tissues are very extensible and that the linear elements are probably greatly folded rather than fully extended. Collagen fibrils exposed to temperatures which cause great contraction in intact tissues show spacings essentially similar to those of unheated fibrils.

All of these observations, together with observations on the relation between the increase of spacing and the applied force, are used for a discussion of the structure of collagen fibrils.

A number of papers have been published in Germany dealing with the structure of cellulose fibres. As many of these papers are at present not available in the United States, a detailed discussion of them must be omitted. Some of them seem to indicate that the concept of the existence of micellar structure must be discounted. The reader is referred to the enumeration of abstracts of these particular papers given together with the original reference in the bibliography [Ruska (114), Ruska & Kretschmer (115), Zahn (116), Mahl (117), Müller (118), Franz, Wallner & Schiebold (119), and Wergin (120)].

As mentioned before, studies of histological nature are very few, and this branch of electron microscopical research can still be considered in its infancy. The closest to histological work which can be mentioned is the observation of relatively rigid structures which can be prepared easily in thicknesses applicable for present day electron microscope requirements. The first of these publications is a short communication by Driest & Müller (121) about observations of chitin-containing objects, in particular, wings and legs of *Musca domestica.* Later a short paper by Frank & Ruska describes the structure of wings of *Pitta maxima* (122).

In direct continuation of this line of research is a very recent paper by Anderson & Richards (123), dealing with "an electron microscope study of some structural colors of insects." The investigation has been carried out on brilliantly colored organs of the blue tropical butterfly, *Morpho cypris,* and the iridescent beetle, *Serica sericea.* For the study of minute structures, stereoscopic observation was used in many instances. They find that

> the iridescence of the beetle, *Serica sericea,* is due to a line grating on its wing covers, 8000 Å between lines. The structure giving rise to the brilliant blue color of the wings of *Morpho cypris* is quite elaborate, consisting essentially of hundreds of vanes on each wing scale, the vanes possessing linear thickenings 200 Å apart, which reinforce the reflection of blue light. Numerous fine markings on parts of the wing scales go down in dimensions to 60 Å, and some of them may be related to the chemical structure of the scales.

The noniridescent wing scales show also very interesting fine structure with striped thickenings about 1500 Å apart. The wing membrane itself also has a short grating-like structure with almost the same spacing between lines.

The authors calculate the spacings which might be expected to produce the brilliant blue color of the *Morpho cypris* and find that the results do not at all check with their electron microscope observations. However, they observed that the position of the specimen was changed, and that the specimen itself appeared to shrink when the full electron beam intensity was turned on it for the first time. "It, therefore, appeared probable that the vanes shrink under intense electron bombardment and that the pictures obtained represent shrunken vanes." Comparative observations of the same specimens under light and electron microscopes supported this assumption. Swelling experiments in alcohol gave evidence that the structures responsible for the color are not destroyed by the electron beam and that they react to swelling agents in much the same manner as unexposed areas. Assuming a proportionality of the swelling of the unexposed areas and of the exposed areas, they find excellent agreement with their calculations of the color from the spacings.

The same authors published two papers dealing with electron microscope studies of insect cuticle (62) and of insect tracheae (63). Of these two papers, that on insect tracheae is discussed here. Tracheae of the larvae, pupae, and adults of the mosquito, *Culex pipiens L.*, air sacs and tracheoles of the adult worker honeybee, *Apis mellifica*, and tracheae of the adult American cockroach, *Periplaneta americana*, were investigated.

> The thickness of intertaenidial membrane of larger tracheae of these species is 100 to 200 Å after drying; it seems probable that it averages less than 200 Å in life. The thickness of the intertaenidial membrane of tracheoles after drying seems to be considerably less than 100 Å and may be only 50 Å.

The tenaedia of mosquito tracheae and in some regions the membrane also

> bear minute spines which are not limited to the region adjacent to the spiracles but occur throughout the length of the longitudinal trunks. The spines are of quite diverse sizes and shapes, the average length is about 1000 Å, with a normal range of 800 to 1500 Å, but much smaller ones also occur with the extremes being mere lumps not over 200 Å.
>
> Most striking in appearance are the bands of large spines that encircle the main tracheal trunk and interrupt the taenidia. These seem not to have been reported heretofore, although they are readily visible in the light microscope.

The determination of the dimensions of these larger spines was made under the light microscope; they are mostly around three microns long. "The tracheae show no pores in the thin membrane" within the

limits of resolution of the electron microscope. The tracheoles of the honeybee, which are "less than 2000 Å in diameter, have a helical taenidium 200 to 300 Å wide. Thus tracheoles, as well as tracheae, are shown to possess supporting taenidia." This latter point is used for a critical discussion of the morphological distinction between tracheae and tracheoles. In view of their findings, the authors are of the opinion that the presence of taenidia in tracheae cannot be used any longer to differentiate between tracheae and tracheoles. "The intertaenidial membrane of the tracheoles is very thin." Without being able to observe its dimensions, the authors estimate that the membrane must be considerably less than 100 Å thick.

Diatoms have been familiar test objects in light microscopy. Different papers contain electron micrographs of diatoms, particularly of *Pleurosigma angulatum* (43, 79, 124). The electron micrographs do not reveal any strikingly new features. For further details we refer to the original papers.

Mention should be made of a paper by Menke (125) investigating the structure of the protoplasm.

Research on chromosomes has not yet met with much success. The difficulties of technique are very great and the interpretation of the electron micrographs is subject to much discussion. For completeness, two publications dealing with electron microscopical observation of chromosomes should be cited: von Ardenne (22) and Clark, Barnes & Baylor (126).

Observation of Molecules

Whereas the observation of virus particles, reported in an earlier chapter, falls well within the scope of "observation of molecules," it seemed worth while to review separately some attempts to observe large protein molecules. A paper by von Ardenne (127) contains electron micrographs of hemocyanin, of edestin, and of glycogen. The observed particle diameters of 220 Å, 80 Å, and 80 Å are more or less in agreement with molecular weight determinations.

Huseman & Ruska report in two papers (128, 129) observations on glycogen and on *p*-iodobenzoyl glycogen. The first of these papers deals with two samples of glycogen, one with an average molecular weight of 1,500,000 and the second with an average molecular weight of 65,000. Whereas the first sample shows particles ranging from 50 to 150 Å units, the particles of the second sample are scarcely discernible. The authors tried, therefore, to observe particles with a higher

contrast and undertook the observation of *p*-iodobenzoyl glycogen, reported in their second paper. They observe that particle size varies between 150 and 300 Å in fair agreement with the computed molecular weight of 6,000,000.

Preparation of bushy stunt virus, edestin, and three different hemocyanins was investigated by Stanley & Anderson (130).

The molecules of bushy stunt virus and of *Limulus polyphemus* hemocyanin appeared to be essentially spherical in shape and to possess diameters of approximately 260 and 200 Å, respectively. Micrographs of *Busycon canaliculatum* and *Viviparus malleatus* hemocyanin showed particles having average diameters of about 220 and 290 Å, respectively.

Evidence is offered that the molecules of the last two hemocyanins are platelike in shape. Edestin micrographs "showed many particles ranging up to about 110 Å in diameter and an almost complete absence of larger particles." The agreement with the molecular sizes estimated by indirect methods can be called fair.

Common to the quoted papers is the fact that in spite of a very good resolving power, no very small organic particles were observed. All the investigations dealing with such small particles are very much hampered by lack of contrast. This is due, as pointed out in the chapter on the mechanism of image formation, to the small number of electrons scattered beyond the solid angle formed by the objective aperture by a thin specimen of low density. It is indeed justified to speak of a "depth resolving power" (35, 131) which is a function of the electron speed, of the density of the specimen, and of the atomic weight of its constituents.

What, then, are our chances of observing organic particles of, let us say, less than 50 Å in diameter. Following Huseman & Ruska's (129) example, we might introduce heavy atoms in the molecule. This, however, is not always possible. Also, we obtain information on a modified particle and not on the unaltered one which we want to investigate.

A very limited remedy is the use of lower accelerating voltages (lower electron speed). Our best chances seem to be in the use of dark-field illumination (16, 21, 47). However, it should be pointed out that up to now, the quality of dark-field electron micrographs is considerably behind that of the bright field images. It is to be hoped that in the near future this condition will be improved and the resolving power of both kinds of micrographs will be made equally good.

This chapter should not be closed without emphasizing that the electron microscope is able to provide us with information about properties of the investigated substances which originally did not seem to be available with this type of instrument. By comparing data obtained from the electron microscope and a series of methods operating through indirect means, one can obtain information about such properties as density of the particles, viscosity of the medium in which they are suspended, asymmetry factors, etc. (131). Also, the limited resolving power of the electron microscope does not mean necessarily that the information gathered is limited to the direct evidence contained in the micrographs. An example might illustrate this point of view. By measuring the width of a great number of ribbon-shaped sodium laurate curd fibres, Marton, McBain & Vold (132) found that these widths are integral multiples of a fundamental unit which could be identified (within the limit of accuracy of the measurements) as twice the molecular length of the sodium laurate. The conclusion that the molecules are oriented across the fibre supports a similar deduction previously obtained by x-ray diffraction methods. However, by examination of the electron micrograph, one can further conclude that the fibres themselves display selective unaxial orientation. The detection and orientation of the micelles has previously been possible only after considerable x-ray investigation.

Emission Microscopy as Applied to Biology

An interesting attempt to use the self-emitting electron microscope for biological purposes was made by Scott and his collaborators: McMillen & Scott (133), Scott & Packer (134 to 137), and Scott (138).

Using a simple microscope described in a paper by McMillen & Scott (133), the technique consisted in microincineration of animal tissues and the use of the electron emission of the remaining ash, containing oxides of sodium, potassium, calcium, magnesium, etc. Their expectation was that when heating these ashes to more or less specific temperatures, they would be able to differentiate between the various elements and localize them in cells. The emission pictures which have been obtained were, however, due to magnesium and calcium alone. They were able to localize these elements in the contraction bands of frozen and dehydrated muscle. They had evidence for concentration of magnesium and calcium in the free border of epi-

thelial cells of the mucous membrane of the stomach and intestinal tract.

This method has the great disadvantage that the resolving power of emission type microscopes is very limited. For instance, in the papers quoted, the best resolving power seems to be of the order of magnitude of several microns. Recent theoretical papers (139) give hope, however, that the resolving power of the emission type instrument can be increased considerably, and there is even one report, of which the reviewer has seen only a brief abstract, indicating that relatively good resolving power has been obtained in Germany (140). Therefore, it seems worth while, in view of these German papers, to consider further studies in that direction.

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THE CHEMISTRY OF VIRUSES

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Although chemists had shown a desultory interest in the biochemical problems posed by viral diseases, the demonstration in 1935 that certain plant viruses are protein macromolecules (1) marked the opening of this field of research to the disciplines of chemistry and physics. An account of the phenomenal progress which was made within the next five years toward an understanding of the nature of these agents was covered by Stanley's comprehensive review in 1940 (2) and by later reviews concerned with special aspects of this rapidly expanding field (3, 4, 5, 6).

The comparative ease with which plant viruses can be obtained in the crystalline state has given chemists working with these agents a considerable advantage over chemists working with the more labile animal viruses. Moreover, animal viruses generally occur in low concentrations in the cells of the host and may possess chemical and physical properties so nearly akin to normal protoplasmic constituents of these cells as to render their separation and purification an arduous task. But as a consequence of the great advance which has been made in an understanding of the properties of plant viruses, there has been, it seems to the reviewer, a too hasty extension of ideas gained from these studies to the formation of over-simplified concepts of viruses in general, and of animal viruses in particular.

Initial publications dealing with the successful isolation, purification, and crystallization of plant viruses led to an immediate invasion of the field of animal viruses with the new techniques and appurtenances of protein chemistry, with the result that there have appeared in rapid succession numerous reports concerning the isolation, from infected tissue, of macromolecules carrying virus activity. The fact, however, that in several instances protein macromolecules, thus far indistinguishable chemically and physically from the viral agents themselves, have been isolated from normal tissues (7, 8) should serve to temper with some caution the ready acceptance, on the basis of evidence thus far produced, that every macromolecule which carries virus activity necessarily represents a virus in its pure state.

Methods of isolation and criteria of purity of viruses.—Methods commonly employed for the determination of homogeneity of large molecules of biological origin have been fully reviewed by Pirie (9). In this review the author examines critically the current concepts of the word "pure" as applied to viruses in particular, and to protein macromolecules in general. Because of the complexity of certain viruses, particularly those belonging to the elementary body group, workers engaged in their purification have not been able to apply successfully the classical tests for purity such as crystallization and solubility. As a consequence, much confusion has arisen as to what other criteria can be relied upon, under these conditions, to yield convincing proof of homogeneity. In the article by Pirie, the limitations of the various techniques which are currently used for this purpose have been critically set forth.

Certain physical methods commonly employed for establishing the homogeneity of protein materials have been applied to vaccinia. Suspensions of elementary bodies, examined under proper conditions, exhibit a single boundary in the analytical centrifuge (10) and in the electrophoresis apparatus (11, 12). It should be pointed out, however, that fulfillment of these criteria alone does not afford adequate proof of purity and that other criteria have been sought. The technique of differential centrifugation, which is generally employed in the purification of animal viruses (13, 14, 15), selects particles with approximately the same rate of sedimentation so that it is not surprising that final preparations display single boundaries in the analytical centrifuge (16). The demonstration that equal mixtures of suspensions of washed elementary bodies of vaccinia and collodion particles, graded by the same technique of differential centrifugation as that used for the virus purification, sediment with a single boundary in the analytical centrifuge, affords convincing evidence of the limitation of this technique when employed as a single criterion of purity (17). A single boundary in the electrophoresis cell is likewise no guarantee of homogeneity since mixtures of particles differing widely in composition and structure, but possessing similar distribution of surface charge, may move in a single boundary (18). Here again it has been possible to show that a suspension of graded collodion particles, coated with the soluble antigen of vaccinia, and a suspension of elementary bodies move in a single boundary in the Tiselius electrophoresis apparatus (17). Obviously the limitations of such physical techniques do not make them valueless but do render hazardous the acceptance of reports

of results obtained by these techniques alone as evidence of homogeneity.

The constancy of chemical, physical, and biological data on many of the viral proteins derived from plants affords adequate evidence that these materials represent viruses in their pure state. Unhappily, the identity of virus and a homogeneous protein with viral activity, obtained from infected animal tissue, is not so evident. The failure to find in normal tissue a homogeneous protein macromolecule, possessing chemical and physical properties similar to that of the protein obtained from diseased tissue, is an important argument for this identity but by no means constitutes adequate proof in itself. Diseased tissues may well possess qualitative as well as quantitative differences in content of protein from that of normal tissue. New protein formed under the complex changes associated with altered metabolism may conceivably bear but an incidental relation to the accompanying viral agent. In a recent report, dealing with the crystallization of an active protein from the brains of mice infected with the virus of poliomyelitis, this point is made quite clear (19).

Studies on the macromolecular protein, isolated by ultracentrifugation from chick embryo diseased with equine encephalomyelitis virus, have revealed the presence of two components. The heavier component ($S_{20^\circ} = ca.\ 250 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}), which carries most of the infectivity, is found only in diseased tissue while the lighter protein ($S_{20^\circ} = ca.\ 70 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}) is similar to a macromolecular protein found in normal chick embryo. In earlier experiments both components appeared equally infectious (8). Separation is claimed to have been achieved by differential extraction of diseased tissues with distilled water, and the infectivity now appears to be almost wholly associated with the heavier material (20). Results of experiments on the preparation of high molecular weight constituents from normal and poliomyelitis-infected medullae cords have been presented by Loring and Schwerdt (21). Normal cord tissue, frozen and stored for several weeks, yielded negligible amounts of sedimentable protein while extracts made from infectious cord material under similar conditions yielded small but definite amounts of a highly infectious macromolecular protein, of which 1 ml., containing 5×10^{-9} gm. of nitrogen, regularly produced poliomyelitis when injected intracerebrally into *rhesus* monkeys. Results of centrifugal analysis showed the presence of a relatively homogeneous protein with a sedimentation constant of $S^{w}_{20^\circ} = 62 \times 10^{-13}$. It is of interest that this value is dif-

ferent from that of $S_{20^\circ} = 160$ to 170×10^{-13} reported by Gard & Pedersen (22). It appears, therefore, that poliomyelitis virus and mouse encephalitis virus differ not only in immunological properties as shown by Theiler (23) but in physical properties as well.

Plant viruses were first isolated by the classical methods of salting out and differential adsorption which had previously been used so successfully in the purification and crystallization of enzymes (24). When it was found that these procedures resulted in the loss of certain physical characteristics of viruses, such as filterability through membranes of small pore size (2) and marked loss of infectivity (25, 26, 27, 28), they were largely discarded in favor of the less drastic method of differential centrifugation (29, 30). Because of the relatively great amount of detritus and bacterial contamination in the crude starting material, it may be advisable in the case of many of the animal viruses to resort to preliminary procedures of adsorption, precipitation, and ultrafiltration in order to achieve a measure of concentration and purification of the virus before employing the technique of differential centrifugation for the final stages of purification (31). By means of adsorption on calcium phosphate or ultrafiltration through collodion membranes, preparation of influenza virus free of 90 per cent or more of the nitrogenous material contained in the crude suspensions of infected mouse lungs has been obtained. When the two techniques are combined, the residual virus represents, in terms of 100 per cent activity, only 1 per cent of the nitrogen content of the starting material. Loss of virus by this method is said to be slight and immunological properties are unimpaired (32).

A new method of virus purification has been reported by Cohen (33). In an attempt to dissociate the nucleic acid of tobacco mosaic virus from its protein carrier by means of heparin, a new type of reaction between very large protein molecules, of which the plant viruses are examples, and hydrophilic colloids, such as heparin, has been revealed. Several proteins of molecular weight greater than 10^6 were precipitated by colloidal substances, and at least two closely related viruses have been separated with relative ease. The isolation of tomato bushy stunt virus and the rapid crystallization of tobacco mosaic virus from infectious juice are described. The precipitation of viruses by heparin at pH 7 does not seem to involve chemical combination.

An important observation made by Hirst (34) and confirmed by McClelland & Hare (35) has shown that influenza virus in the chorio-

allantoic fluid of chick embryos can be adsorbed by erythrocytes and that the virus once adsorbed can be readily eluted at temperatures between 22° and 37° (36). Hirst and his co-workers have utilized this phenomenon to devise a simple method for the quantitative determination of influenza virus and influenzal antibodies (37) which seems to offer decided advantages over the mouse test originally employed for this purpose. Hirst and co-workers have also shown that the precipitate which forms when infected chorioallantoic fluid is frozen and thawed contains most of the active influenza virus and have used this technique for the concentration of the active agent (38). Francis & Salk (39) have extended the studies of Hirst and have utilized the phenomenon of erythrocyte adsorption for the concentration and purification of influenza virus from the chorioallantoic fluid of the infected chick embryo. By this method a tenfold concentration of the virus has been obtained consistently with consequent elimination of the major portion of the inert chick protein. These workers consider this method of adsorption and elution from embryonic red cells as effective as precipitation in concentrating the virus with the added advantages of avoiding the technical difficulties inherent in the procedure of precipitation.

Composition and structure.—Until recently the attention of the chemist working with virus constituents has been directed chiefly toward studies of the physical and chemical properties of the nucleic acids in viruses. With the possible exception of coliphage (40), none of the twenty or more purified viruses thus far has been obtained free of nucleic acid. Even in purified coliphage, the minute amount of phosphorus present may conceivably have come from traces of nucleic acid. Otherwise, viral agents may contain from 5 to 40 per cent of this "common denominator" substance. With the exception of the viruses of psittacosis and vaccinia (41), all viruses thus far reported have given positive tests for pentose and negative tests for desoxyribose and probably contain nucleic acids of the yeast variety (16, 28, 42, 43). Except for tobacco ring spot virus, of which a portion of the nucleic acid can be removed by salt dissociation, the nucleic acids in viruses appear to be firmly bound and require strong alkali in order to effect separation from their protein components.

It is well known that a given virus may exist in different though immunologically closely related forms. Examples are known of direct conversion of one strain into another (44). The high molecular weight nucleoprotein from the yellow aucuba strain of tobacco mosaic

virus possesses properties very similar to those of a closely related strain of ordinary tobacco mosaic virus. Small differences in properties have been found, however, so that it is possible to mix the two nucleoproteins and separate them again into two fractions, one carrying tobacco mosaic virus activity and the other aucuba mosaic virus activity. Pfankuch (44) has drawn the general conclusion, on the basis of results obtained from electrophoretic studies of the protein components of two closely related strains of virus, that differences between tobacco mosaic virus strains can best be explained by quantitative differences in the nucleic acid components. Nucleic acid was removed from electrophoretically homogeneous preparations of tobacco mosaic virus, and of a variant, tobacco mosaic virus 44, by means of alkali or pyridine, and the proteins were studied in the Tiselius apparatus. Each protein was reported to be electrophoretically homogeneous and when mixed gave a single boundary although the intact nucleoproteins when mixed gave a double boundary. On the basis of this information, test of solubility, and an earlier report of marked differences in phosphorus content of the two strains (45), the conclusion was reached that the protein components were identical and that the nucleic acids were different. Evidence leading to a contrary conclusion has been advanced by Knight & Stanley (46) who maintain that in spontaneously arising strains of tobacco mosaic virus and in a rib grass strain of tobacco mosaic virus the chemical differences between strains lie not in the nucleic acids but in the protein parts of the virus molecules. Proteins from ordinary tobacco mosaic virus and from the rib grass strain of tobacco mosaic virus were freed of nucleic acid by alkali and studied in the Tiselius apparatus. A mixture of these proteins at pH 7.1 and pH 10 migrated in an electric field as if composed of one component although, by chemical analysis, important differences in their amino acid compositions were observed (18). From these experiments it was concluded that the electrophoretic technique could not be relied upon to distinguish between biologically related but chemically different proteins. A study of the differences in amino acid contents of closely related strains of tobacco mosaic virus protein has served, it would seem, to re-emphasize the role of the aromatic amino acids as determinants of immunological specificity (42, 47).

The plant virus proteins afford a unique opportunity to study the relationship between structure and immunological specificity because frequent variations occur, resulting in the production of new strains

of viruses which possess properties quite distinct from those of the original agent. Recently, mutations induced in tobacco mosaic virus as a result of the treatment with x-rays and γ-rays have been reported (45). Serological tests with six strains of tobacco mosaic virus, cucumber viruses 3 and 4, and tobacco mosaic virus antiserum showed a close relationship between tobacco mosaic virus and all the viruses tested with the exception of the rib grass strain and cucumber viruses 3 and 4. Analyses of 12 preparations of tobacco mosaic virus revealed the presence of 3.8, 4.5, and 6.0 per cent of tyrosine, tryptophane, and phenylalanine, respectively (46). Results of analyses on yellow aucuba, green aucuba, and two additional strains of tobacco mosaic virus were the same as for ordinary tobacco mosaic virus. However, pronounced differences in tyrosine, tryptophane, and phenylalanine values were found for the rib grass strain of tobacco mosaic virus and cucumber viruses 3 and 4. Ross (48) has reported that tobacco mosaic virus contains 3.9 per cent tyrosine, 4.5 per cent tryptophane, 4.7 per cent proline, 9.0 per cent arginine, 6.7 per cent phenylalanine, 6.4 per cent serine, and about 5.3 per cent threonine. Glycine and histidine appear to be absent. No chemical differences in amino acid composition were noted between chemically isolated samples and those isolated by ultracentrifugation; this fact is interpreted as showing that the samples were essentially pure. A cysteine content of 0.68 per cent for tobacco mosaic virus (49) is interesting because if virus is made up of uniform subunits, as suggested by Astbury (50), the smallest possible uniform unit, based on the cysteine analysis, would amount to a subunit molecular weight of 17,800 which is considerably larger than that proposed by Astbury (48). To the known amino acids in tobacco mosaic virus we may now add glutamic acid, 6.3 per cent; aspartic acid, 2.4 per cent; leucine, 6.1 per cent; valine, 3.9 per cent; and alanine, 2.4 per cent; these bring the total quantity of the known constituents of the virus to about 68 per cent (51).

Although the isolation, purification, and crystallization of the plant viruses have engaged much of the chemist's attention during the past several years, it should be pointed out that considerable progress in the purification of at least one animal virus, the virus of vaccinia, had been achieved by the time the crystallization of tobacco mosaic virus was reported (52, 53, 54). The relatively enormous size of the elementary body of vaccinia and its exceptional resistance to physical and chemical change have made it fairly easy to obtain sufficient quantities of this virus for studies of its chemical, physicochemical, and bio-

logical properties (55, 56, 57). In the hands of Rivers and his associates (58) the technique of purification has been perfected until it is capable of yielding elementary bodies of vaccinia, which on the basis of consistent activity and homogeneity, may be claimed to satisfy many of the criteria laid down by protein chemists for chemical purity. Elementary bodies prepared by differential centrifugation give constant analyses for nitrogen, phosphorus, lipid, and carbohydrate. Analyses for basic constituents performed on eleven lots of elementary bodies gave an average value of phosphorus, 0.57 per cent, nitrogen, 15.3 per cent, total lipid, 5.7 per cent, and reducing sugars, 2.8 per cent (59). In a classification of viral agents according to size, beginning with the virus of foot and mouth disease, bacteriophage, and the plant viruses at the lower extreme and with rickettsiae and smaller bacteria at the upper extreme, the elementary body of vaccinia is seen to fall much nearer the frankly organized agents such as rickettsiae than to the smaller viruses, such as tobacco mosaic virus (60). Because of this fact and the chemical composition (59, 61, 62), the concept of viral agents as protein macromolecules, while valid for many of the plant viruses, is hard to accept when forced on the more highly organized and complex agents such as those causing vaccinia, psittacosis, and infectious myxomatosis of rabbits. Considerable interest has been shown in the lipid content of elementary bodies of vaccinia because if lipid is an integral part of the virus, then the molecular concept of viruses cannot be applied to the agent causing vaccinia unless it is assumed to be a complex lipoprotein macromolecule, an hypothesis which robs the term "molecule" of much of its classical meaning (16, 62). Lipid in elementary bodies of vaccinia is not hydrolyzed by lipase but is readily digested by lipase following its extraction from the virus by alcohol and ether. Fractionation of this lipid reveals cholesterol, 1.4 per cent, phospholipid, 2.2 per cent, and neutral fat, by difference, 2.2 per cent. The cholesterol can be removed by cold extraction with ether without impairing the activity of the virus and is, therefore, not considered an essential part of the viral agent (59). The role of lipids in the surface structure of elementary bodies of vaccinia cannot be assessed at this time. Electrophoretic mobility of the virus, like its infectivity (12, 59, 62), is not affected by the removal of cholesterol by ether extraction. Whether phospholipid and neutral fat, not removable from elementary bodies by this treatment, are involved in the limiting membrane encasing the particle and responsible, perhaps for its osmotic responses, has not been determined.

The nucleic acid of elementary bodies of vaccinia appears to be of the thymus variety (41, 63). Nucleic acid obtained from elementary bodies of vaccinia by alkaline extraction gave an elementary analysis comparable to that calculated for a tetranucleotide, a strongly positive Feulgen reaction, and an intense color with diphenylamine. Crystalline adenine and guanine were recovered from the material, and qualitative tests for thymine were positive. An ultraviolet absorption spectrum was characteristic for nucleic acid. No effect on the virus nucleic acid by ribonuclease could be detected (41).

With respect to the protein component of vaccine virus, particularly in regard to its amino acid composition, no important work has yet been reported.

Protein-free ribonucleic acid has been isolated from heat denatured tobacco mosaic virus and subjected to electrophoresis and to studies of partial specific volume, diffusion, sedimentation, viscosity, and osmotic pressure. Studies of the ribonucleic acid by means of electron microscopy were made, and analytical values given for its elementary composition (64). Freshly isolated nucleic acid has an average particle weight of 300,000 and is highly asymmetric. With spontaneous decomposition it forms particles with molecular weights of approximately 61,000. Both molecules appear to be too long to lie within the 150 Å length of the virus molecule. The nucleic acid may be converted by means of cold alkali to particles having a molecular weight of 15,000 and an axial ratio of 10.

Chemical derivatives of tobacco mosaic virus.—A unique attack on the problem of the relationship of chemical structure to the biological activity of the plant viruses has been launched by Miller & Stanley (65). With the idea of learning what effect a chemical derivative of an infectious virus might have on host cells and on the type of virus subsequently arising from a virus inoculum which had been chemically altered, acetyl and phenylureido derivatives of tobacco mosaic virus were formed. These were inoculated into susceptible hosts, the resulting crop of virus was isolated, and studies were made of its chemical and physical properties. Biological tests showed that the specific activity of virus, in which 70 per cent or less of the amino groups were covered, was the same as that of the untreated virus. When amino groups were 75 to 85 per cent acetylated, there was 25 to 50 per cent inactivation. A preparation, of which 70 per cent of the amino groups and 20 per cent of tyrosine plus tryptophane groups were converted into acetyl and phenylureido compounds, respectively,

was inoculated into young tobacco plants. The disease which was produced was indistinguishable from that caused in the control plants inoculated with untreated virus. Identity of virus resulting from acetyl and phenylureido inocula with that obtained from controls was revealed by the comparable yields and in the chemical and physical properties of the two virus preparations. These experiments are interpreted as suggesting that infecting virus molecules may not necessarily function as exact patterns for reproduction, and as showing that a large portion of certain functional groups of the virus molecule may be altered without interfering appreciably with virus activity as reflected in the type of disease produced and in the physical and chemical properties of subsequent generations of virus (66). Further evidence that tobacco mosaic virus can be altered structurally without altering its activity, and without perpetuation of these induced structural changes in subsequent generations of virus, is provided by the work of Anson & Stanley (67). Inoculation of tobacco plants with active virus, whose sulfhydryl groups had been abolished by iodine, resulted in the production of virus with the normal number of sulfhydryl groups. When sufficient iodine was added or when the iodine reaction was carried out at a higher temperature, the tyrosine groups were converted to diiodotyrosine and the virus was inactivated.

Molecular size and shape of purified viruses.—Studies on an infectious protein isolated from papillomas of rabbits (68) have been extended. Reports are now available on its molecular size, shape, homogeneity (69), and electrophoretic behavior (70 to 74). Investigations of this protein are interpreted to show that it possesses the biological properties ascribed to rabbit papilloma virus, as demonstrated by quantitative studies on infectivity and by immunological or serological tests. No trace of the protein has been obtained from noninfective warts from domestic rabbits. The molecular weight, calculated from sedimentation and diffusion constants, is 47,100,000. Evidence is furnished that the protein resembles an oblate ellipsoid with axial ratio of about 11 if hydration is neglected, and 7 if 33 per cent hydration is assumed. The results show that the papilloma protein is well suited to studies in the electrophoretic apparatus. The protein exhibits a high degree of electrophoretic homogeneity on both sides of the isoelectric point (70). The papilloma protein migrates with a single boundary at all pH values in its range of solubility and biological stability (69).

The sedimentation constant of centrifugally purified bushy stunt

virus in phosphate buffer, at pH 7, is now placed at 131.6×10^{-13} (75), which checks closely with values obtained on a single preparation in five other laboratories. This value differs materially from a sedimentation constant of 146×10^{-13} reported previously from another laboratory (76). The diffusion constant of tomato bushy stunt virus is placed at $D_{20°} = 1.15 \times 10^{-7}$ (77). From this value and the sedimentation constant, a molecular weight of 10,600,000 and a dissymmetry constant of $f_d/f_o = 1.27$ have been obtained. This corresponds to a molecular axial ratio of 5.4/1 and 5.8/1 for prolate and oblate ellipsoids of revolution, respectively, if hydration is assumed to be negligible.

A minimum condition which a virus protein must satisfy in order to be considered molecular, if our classical concepts of homogeneity are to be retained and extended to the protein macromolecules, is absolute homogeneity with respect to size and shape. To demonstrate homogeneity by means of the ultracentrifuge it is necessary to show that observed boundary spreading can be entirely accounted for on the basis of the diffusion rate of the material. Lauffer (78) has subjected the virus of bushy stunt to critical tests for inhomogeneity. Bushy stunt virus is known not to possess sufficient degree of inhomogeneity to be detected by means other than ultracentrifugation, and since the particles are approximately spherical, it is a favorable material for tests of inhomogeneity in the ultracentrifuge. Accordingly, the spreading of a bushy stunt virus boundary in the ultracentrifuge was measured and compared with the theoretical spreading calculated from known diffusion constants. These studies have shown that the boundary spreading can be accounted for entirely in terms of the calculated diffusion. In view of this result it has been concluded that molecules of bushy stunt virus are strikingly homogeneous with respect to size, shape, and density and therefore fulfill the most exacting physical criteria for protein molecules.

Somewhat startling values for the size of unpurified influenza virus A have been reported by Bourdillon (79) who claims to have demonstrated a fast diffusing fraction of the virus with a particle size of 6 mμ. This value for influenza virus is at such variance with that of 100 mμ found by other workers by techniques of ultracentrifugation and ultrafiltration (80, 81, 82), as to make for cautious acceptance of this new data until the studies have been repeated by other groups with other techniques of diffusion. Preliminary results are also reported to show that in vaccine virus as well, there is a small, rapidly

diffusing fraction. Neurath (83) in a comprehensive review has called attention to the limitations of the technique employed by Bourdillon (84) as well as to certain other methods commonly used for the investigation of protein diffusion.

Electron microscopy of viruses.—Theories concerning the physicochemical behavior of spherical particles have been worked out with reasonable thoroughness and appear to fit most of the observed facts relating to spherical molecules of biological origin (78). Unfortunately, the situation is not so clear for particles which depart markedly from the spherical form. The technique of electron microscopy came (85 to 89), therefore, at a time when it could be of great service to chemists concerned with the physical treatment of these substances. Purified preparations of five viruses have been studied by means of the electron microscope (90). Electron micrographs of tobacco mosaic virus showed a predominating unit of 280 mμ in length, together with end-to-end and side-to-side aggregates of this unit. These dimensions were of the same order of magnitude as those previously estimated by indirect methods. Electron micrographs of cucumber mosaic virus 3 and 4 were very similar and of a size and shape not greatly different from that of tobacco mosaic virus. Micrographs of tomato bushy stunt and tobacco necrosis virus showed spherical particles of about 26 mμ and 20 mμ in diameter, respectively. The fact that data from electron micrographs agree closely with those obtained by more indirect means gives significant validity to these latter methods when properly applied. Micrographs of a mixture of tobacco mosaic virus and rabbit antisera show increase in particle width from 15 mμ to 60 mμ and in length from 280 mμ to 300 mμ (91). This increase in particle width and the appearance of fuzzy profiles of virus-antisera particles are taken to indicate that ends of asymmetrically shaped molecules from the serum react specifically with the virus antigen. No reaction between virus and normal heterologous antiserum was demonstrable (92).

Electron micrographs of elementary bodies of vaccinia have been particularly helpful in gaining a notion of their internal structure and of providing a new criterion of purity (85, 93). When examined under proper conditions, elementary bodies show six rectangular plane surfaces, arranged in a brick-like manner, and five areas of condensation which appear darker than the surrounding substance (94). The centrally placed area of condensation is larger than the four areas of approximately equal size which are placed around it. No comparable

patterns were encountered in control materials. Elementary bodies readily absorb proteins (12, 95), and if these are not removed, the particles appear oval in outline and are of homogeneous density (85). Specific antibody, unlike other adsorbed serum proteins, is tightly held even after careful washing. Swelling and rupture of elementary bodies treated with alkali are readily demonstrable by electron micrography. These observations clearly indicate that elementary bodies of vaccinia have a characteristic morphological structure, similar in many respects to that of bacteria (96, 97, 98) and differing markedly from that of plant viruses (83, 87). The nature of the areas of condensation in vaccinia virus, like those in bacteria, is not known. In the latter it is thought to be chromatin (99).

Studies begun by German workers on the electron microscopy of phage particles (100) have been extended in this country by Luria & Anderson (101). Although the original reports described phage as round particles without much definite structure, Ruska (102) has recently shown pictures of phage as "sperm-shaped" particles adhering to bacterial membranes. The possibility that these particles might represent bacterial products and not true phage was entertained. Micrographs made by Luria & Anderson of high titer suspensions of bacteriophage, anti-coli BC, have shown consistently the presence of particles of constant and characteristic morphology. These consist of a thin "tail-piece" and a much thicker "head," the latter showing an internal structure of granules distinguished by high electron-scattering power. The "head" diameter appears to be about 80 mμ and the "tail" about 130 mμ in length. When allowed to stand in the presence of sensitive bacterial cells, the particles are readily absorbed and appear to stick to the bacteria by the head or tail. Bacteriophage, anti-coli P 28, and anti-staphylococcus 3 K have also been photographed and appear to differ sufficiently in morphology to allow individual recognition by this technique.

X-ray studies on viruses.—Before 1936 it was assumed that viruses were, in general, incompressible spheres and all calculations were made on this basis (103). Recent examination of the optical properties of purified preparations of tobacco mosaic virus has shown these assumptions to be invalid and the virus particles to be anisodimensional. Analysis by x-rays has shown that rods of tobacco mosaic virus are at least ten times as long as they are wide. Work on sedimentation, viscosity, diffusion constants, and recently with the electron microscope has amply confirmed ideas of the size and shape

first gained by crystallographic studies. Tobacco mosaic virus solutions of sufficient purity have been separated into two liquid layers (104, 105) with the denser layer, formed by the fusion of tactoids, exhibiting the greater purity. An explanation of these observed phenomena is offered by Bernal & Fankuchen (106, 107) who suggest that the cusps of the fused tactoids are occupied by particles of impurities. X-ray measurements made on dried preparations and on solutions of various concentrations showed that, at the same pH, spacings between the virus particles increased with increasing dilution. X-ray patterns have revealed certain unsuspected irregularities and show that distance between rods is inversely proportional to the square root of the concentration by volume. Particles are distributed in a hexagonal lattice or pseudohexagonal lattice of dimensions, $a = 87$ Å, $c = 68$ Å, and fill all available space as uniformly as possible. The particles appear equidistant and parallel but show no regular arrangement in the direction of rod length. Patterns made by high angle photographs of the internal structure of tobacco mosaic virus show an internal regularity similar to that of crystals. Components of particles, as well as particles themselves, appear regularly arranged. The virus molecules seem to be composed of submolecules with dimensions of 11 Å cube. With respect to internal structure, the virus molecule appears to be much more complex than the protein myosin but probably much less complex than crystalline pepsin. Internal regularity (108), chemical simplicity, and a lack of water even when particles are in solution would seem to separate the crystalline plant viruses most sharply from the simplest organisms higher in the virus scale. Studies on strains of tobacco mosaic virus, on potato virus X, and the virus of bushy stunt show a distinct pattern for each virus with detectable differences between closely related strains of the same virus (107). This would indicate that serological differences may be explained in part on orientation in space of the virus molecule as well as on certain differences in amino acid composition. Thus, with the newer technique of x-ray analysis and electron microscopy (88, 89), agents of particle size ranging from bacteria to atoms have become capable of direct optical examination.

Antigenic structure of viruses.—In contradistinction to the simple antigenic pattern of the plant viruses and of at least one animal virus (that causing papillomatosis of rabbits), a number of animal viruses, such as those of vaccinia, yellow fever, psittacosis, myxomatosis, influenza, and lymphocytic choriomeningitis, possess more complex

antigenic structure and give rise to more than one specific antibody (16). Of the various antigens, those of vaccinia have been most thoroughly studied. Four distinct antibodies to the antigens in vaccinia have been demonstrated in hyperimmune sera by *in vitro* techniques: *e.g.*, L, or heat labile antigen; S, or heat stable antigen (109); NP, or nucleoprotein antigen (110); and an as yet uncharacterized X-antigen (111). Neutralizing antigen is distinct from these and probably from X-antigen as well (16). Two of the antigens responsible for the stimulation of antibodies have been obtained in purified forms and their properties have been carefully studied. That L and S represent a single substance with different antigenic groups has been demonstrated by Shedlovsky & Smadel (112) by the isolation of a single protein molecule which possesses all the serological properties attributed to both the L- and S-antigens. Serological, chemical, and physical properties of this substance have been reported. The LS-protein is characterized as follows: density, 1.39; specific volume, 0.72; diffusion constant, 1.5×10^{-7}; sedimentation constant, 4.3; isoelectric point, pH 4.8; nitrogen, 15.7 per cent; and negative tests for phosphorus, lipid, glucosamine, and nucleic acid. LS-protein provides an example of a single molecule that is capable of eliciting two distinct antibodies. It is of interest, too, that either of the two distinct antigenic properties can be altered selectively by means of heat and alkali (112), which leave the stable antigen intact, or by crystalline chymotrypsin, which destroys the stable component (113) and leaves the labile antigen intact. The chymotrypsin-treated antigen, carrying L-antigenic properties, has been crystallized (114). The nucleoprotein antigen of vaccinia makes up an appreciable portion of the total mass of the elementary body (62, 63). Immunological data show that the nucleoprotein is a distinct antigen but that it is not the substance responsible for immunity or the neutralizing antibody. Analysis of this nucleoprotein reveals nitrogen, 14.5 per cent, phosphorus, 1.8 per cent, and nucleic acid of the thymus type.

A close relationship between infectivity of the purified rabbit papilloma protein and its complement-fixing power has been reported by Beard and his co-workers (115). The mean quantity of protein required to fix one unit of complement was found to be about $10^{-6.023}$ gm. Correlation of infectivity measurements with the purity of this protein has been reported (116, 117) and quantitative studies have been made on the relation between serum and total and free virus (118, 119).

Studies on virus metabolism.—Knowledge of the intracellular nature of viruses has led to considerable speculation concerning the biochemical and physiological relation existing between the viral agent and the host cell. Does the virus depend wholly for its substrate on the cell, or in more highly organized viral agents are there fragments of rudimentary systems which are capable of some degree of independent metabolic activity? In the case of the highly organized vaccine virus, the hypothesis has been made that as an obligate parasite it may possess only a portion of the enzyme and redox systems necessary for survival, relying chiefly upon the intracellular milieu for complementary systems which enable it to multiply within the host cell (120). Parker & Smythe (121) could demonstrate no independent metabolism of purified elementary bodies of vaccinia by oxygen utilization or acid production in the presence of hexose phosphate and respiratory factor from red cells. However, Hoagland and associates have reported finding substantial quantities of copper (120), flavin-adenine dinucleotide (122), and biotin (123) in purified elementary bodies of vaccinia. These substances appear to be present in constant amounts in their most highly purified preparations and are not removed by frequent washing over a wide pH range, by ultrafiltration, or electrodialysis. Macfarlane & Salaman (124) have reported the presence of phosphatase and catalase in elementary bodies of vaccinia but were unable to demonstrate dehydrogenase activity. The virus preparations were also reported to possess phosphomonoesterase and phosphodiesterase activity and an enzyme which hydrolyzed adenylic acid (125). Certain of these studies have been repeated and extended to other enzymes which are found in the tissues from which the virus is prepared (95). At the same time tests were made of the adsorptive capacity of elementary bodies for these enzymes. Lipase, catalase, and phosphatase were adsorbed by the purified virus from dilute solutions of the enzymes with great rapidity and could not be eluted by repeating the washing procedure used in the initial preparation of the virus from infected tissue. On the basis of these results it was concluded that no distinction could be made between the enzymes which may represent an integral part of the virus and those which exist as contaminants from the host. No convincing reports on enzymes of the plant viruses have appeared. The absence of important quantities of biotin, riboflavin, and pantothenic acid in repurified tobacco mosaic virus has been reported (126).

Delbrück & Luria (127) have elaborated a theory which purports

to account for the mechanism of growth of bacteriophage and the nature of interferences between "bacterial viruses." Quantitative studies on the nature of the growth of two phages upon a single bacterial host are reported. Phage is considered as being produced by the intervention of a "key-enzyme" present in limited quantity in each bacterial cell. Interference between certain bacteriophages and interferences between particles of the same phage are attributed to competition for this hypothetical key-enzyme. Analogies are drawn between the interference phenomena among the bacterial viruses and similar phenomena known to occur among certain plant (128) and animal viruses (129, 130, 131).

Effects of enzymes and reagents on purified viruses.—Studies on the physical properties of elementary bodies of vaccinia, on their resistance to autolysis and on their maintenance of intact structure even after prolonged storage in the cold, have revealed a stability not shared by most other animal viruses (16). Merrill (132) has reported studies of the effects of crystalline proteolytic enzymes on elementary bodies of vaccinia. Pirie (133) has shown that many of the lethal effects on certain viruses formerly attributed to pancreatic enzymes are due to fatty acids which these preparations contained and not to specific enzymatic activity. Hoagland and co-workers (134), in a survey of the effects of various proteolytic enzymes on purified elementary bodies of vaccinia, found no immediate effect of crystalline trypsin, crystalline chymotrypsin, or crystalline carboxypeptidase on the virus at physiological pH and temperature. Purified cathepsin was likewise ineffective. Pepsin, at a pH which in itself inactivated the virus, resulted in a rapid release of free amino groups. Papain–hydrogen cyanide mixtures, over a pH range compatible with virus activity, however, effected within two hours marked change in the structure of elementary bodies with complete destruction of virus activity.

Much uncertainty exists as to the true nature of the changes associated with loss of viral activity that results from the experimental treatment of viruses with various deleterious agents. A clue to the nature of certain of these changes may be contained in the results of experiments on the reactivation of inactivated plant and animal viruses and of bacteriophage (135). Johnson (136) has studied the influence of various chemicals on the infectivity of tobacco mosaic virus by means of an agar-disc technique. This method permits the diffusion of chemicals to the virus when held in the agar and the subsequent

extraction of the chemicals with distilled water. In this manner it is possible to dissociate the primary effect of the reagent on the virus from a possible secondary effect of the reagent on the host cell. No evidence was secured to indicate that trypsin or other substances tested modify the host plant, rendering it less susceptible to infection. The suggestion is made that trypsin and certain other substances inactivate the virus through the formation of a relatively loose specific molecular union, which can be broken up by removal of the inactivator by water extraction, provided no chemical destruction of the virus has occurred. Heretofore, results of studies on the reactivation of tobacco mosaic virus have retained a distinct element of uncertainty because of the belief of Stanley and associates (137, 138) that the effect of the reagent on the host cell is in some manner connected with the results obtained with virus inactivation.

The studies of Loring (139), who has reported a reversible inactivation of tobacco mosaic virus by crystalline ribonuclease, have thrown considerable light on the type of combination which may occur between the protein of the virus, its nucleic acid, and the nature of the enzymic action. Inactivation of the virus with this enzyme appears to be comparable to the reaction given by other proteins with high isoelectric points and probably occurs as a result of the formation of complexes between oppositely charged particles, like those reported for the action of clupein and papain on tobacco mosaic virus and the reaction between ovalbumin and nucleic acid described by MacInnes & Longsworth (140). The complex formed between ribonuclease and tobacco mosaic virus, unlike the ovalbumin–nucleic acid complex, is insoluble in water and gives characteristic crystals or fibers. On analysis these fibers showed 14 per cent ribonuclease and could be readily broken up to yield free, active virus.

Schramm (141), as a result of studies of the action of intestinal nucleophosphatase on tobacco mosaic virus, has claimed that nucleic acid could be completely removed from the virus, leaving a protein whose physical characteristics, as determined by size, homogeneity in the ultracentrifuge, and electrophoretic mobility were the same as those of the original protein. Cohen & Stanley (142) have not been able to confirm these reports. In their hands an enzyme prepared from the intestinal mucosa of calves, which possessed marked hydrolytic activity for yeast and virus nucleic acids, was found to have no demonstrable effect whatever on tobacco mosaic virus when tested under a variety of conditions.

Report of a factor in domestic rabbit papilloma tissue which hydrolyzes the rabbit papilloma virus protein has been reported by Bernheim *et al.* (143). The presence of this agent in domestic rabbit tissue is offered as an explanation for the puzzling observation that a low titer or absence of infectious agent occurs in extracts from papilloma of domestic rabbits, in contradistinction to high titers of infectious agent which can be obtained in extracts made from the papilloma of the wild or cottontail rabbit. Because of previous explanation of this difference between domestic and wild rabbit papillomas on the basis of a specific antigen against the virus in tumors of the domestic rabbit (144), the work of Bernheim *et al.* requires confirmation and extension before the results can be accepted as the full explanation of this interesting example of differences in the host-virus relationship between two species of animal.

Francis (145) has reported the presence of a factor in nasal secretions which is effective in inactivating the virus of influenza. Nasal secretions with the highest titers of this factor were obtained from individuals with high titers of serum antibody while weaker concentrations tended to be associated with sera of lowest titer of influenza antibody. Properties given for this factor are those ordinarily associated with antibodies rather than with enzymes (146).

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MICROCHEMISTRY

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The atomic concept of matter and the law of chemical equilibrium imply that chemical processes should not be affected by the scale on which they are carried out. This should hold at least as long as the amount of reacting matter does not rather closely approach molecular dimensions, i.e., remains above 10^{-18} to 10^{-20} gm. If the surface has anything to do with the reaction, one may expect, however, that variation of the volume of a homogeneous system will produce consequences since the surface area does not change in direct proportion with the volume. With heterogeneous equilibria an effect of the relatively increased area of contact must be expected when working with small quantities. Furthermore, the separation of a new phase may be delayed if the number of reacting molecules is small and effective collisions occur infrequently. Because of these uncertainties, chemical reactions on a very small scale, as described by Chambers (1), were performed by Rachele (2), and Loscalzo (3) under conditions permitting close control of the volumes and the concentrations of the reacting solutions. Drops of test solutions and reagent solutions were brought together in the microscopic field by means of micropipettes operated by micromanipulators, and qualitative phenomena customarily observed in test tubes were obtained in drops of 10^{-9} to 10^{-10} ml. volume containing 10^{-12} to 10^{-14} gm. of tested-for ion. Still smaller quantities could have been manipulated, but the above limits were set by the impossibility of perceiving satisfactorily the outcome of the tests. The conclusion appears justified, however, that chemical behavior remains essentially unchanged when only 10^{-8} to 10^{-10} gm. of matter are used. This safeguards the present-day range of chemical microtechnique usually applied to quantities from 0.01 μg. to 50 mg. of material.

Preparatory Work

Preparatory work on a small scale is mostly needed for the final purification of isolated substances, previous to determination of physical constants or ultimate chemical analysis. At times the isolated

substance is either difficult to purify or its physical constants are poorly defined or insufficiently characteristic. It also may happen that the ultimate analysis is unable to reveal the exact composition because of insufficient precision of the methods of determination. It is then necessary to prepare more suitable derivatives. Finally, if molecules are to be marked by tracer isotopes available only in minute quantities, the use of microtechnique in the preparation, isolation, or purification of the compound may offer a solution of the problem (4).

Depending upon the particular process to be carried out, preparatory work may be feasible with a few micrograms of material. The techniques of filtration, extraction, distillation, and sublimation developed for the purposes of qualitative analysis may be utilized. For the most part, however, synthesis or purification of organic substances is performed on a relatively large scale so as to obtain at least 5 to 20 mg. of the substance for analysis. A considerable number of apparatus and procedures for this type of work have been suggested in recent years. The advantages of centrifugal draining in the separation of mother liquor from crystals were demonstrated by Richards (5) several decades ago. Some more microdevices for the performance of this operation have now been described by Craig (6) and by Johnson & Miller (7). The little known, extremely simple artifice of Kunz (8) may be used to advantage for the centrifugal drainage of crystals on a very small scale: one end of a capillary is drawn out so fine (0.01 mm. bore is the practical lower limit) that the crystals are unable to pass through. For centrifuging, the capillary is mounted in the opening of a microcone by means of a split cork.

A combination of separatory and sedimentation funnel has been described by Alber (9). The cavity in the stopcock provided for the collection of sediment is also useful in the isolation and purification of small volumes of heavy liquids. The stopcock has been given an average diameter of 20 mm. to make the use of grease unnecessary except at the two extremities of the ground surface. An ether-insoluble stopcock lubricant has been described by Herrington & Starr (10).

The method of Emich for the fractional distillation of small volumes of liquids has been refined by Morton & Mahoney (11); it is now possible to obtain 30 to 70 fractions when starting with a drop of approximately 25 mg. mass. The fractionation is performed in a capillary of 1.5 to 2 mm. bore and 130 mm. length, which is packed with glass wool nearly to the top. For heating, the capillary is placed

into the well of a copper block after slipping an insulating jacket, consisting of glass or asbestos tubing, over the central portion of the capillary. Thus, heat is supplied at a lower rate to that part of the capillary which acts as the fractionating column. The process of fractionation requires repeated, careful heating and centrifuging, and finally the fractions are collected in capillary pipettes, the diameter of which may be as small as 0.2 mm. in the wide part. It is possible to collect fractions of approximately equal volume. After determination of the boiling points of the consecutive fractions by the method of Emich, the boiling temperatures may be plotted against the fraction numbers. Save for constant-boiling mixtures the graph will show the presence of several components and indicate, if reasonable care has been exercised, the percentage composition of the mixture. Graphs demonstrating the results obtained with mixtures of benzene-toluene, benzene-xylene, acetone-alcohol, ethyl alcohol-*n*-butyl alcohol, ethyl acetate-butyl acetate, and benzene-xylene-butylbenzene are given in the paper. It is obvious that after determination of the boiling points fractions may be further investigated individually or combined for further treatment and investigation.

A critical investigation of existing apparatus for micro extraction was carried out by Batt & Alber (12). They find among others that for highest efficiency the sample should be extracted in a platinum container with a filter layer of platinum-iridium sponge. The solvent should percolate through the material. The receiver for the extract should be light in weight and preferably of platinum, and the ratio of total solvent volume to the solvent capacity of the thimble should be 50:1, with the ratio of the volume of the whole extraction chamber to initial volume of solvent as small as practical. A tubular, hinged electric heater to envelop the sides of the extractor or for the heating of ground glass joints of the extractor is described. Heating from above by means of a Chromolox ring is recommended for the evaporation of the extract.

Apparatus for the extraction of lipids from wet tissues has been described by Holmes (13). The moisture is driven off in the presence of the solvent and its vapor, and collected in a trap inserted between the condenser and the sample. The extraction is more nearly complete in this manner than when the specimens are previously dried or remain wet throughout the extraction.

The drying of substances is usually the last step of preparatory work, and the treatment of small amounts of hygroscopic solids and

liquids has been carefully studied. For the drying and weighing of solids, Alber (14) describes a charging tube and a metal rack for holding it in the drying pistol. The charging tube can be closed inside the drying chamber so that its contents do not come into contact with the atmosphere. Inorganic hydrates, purine and pyrimidine derivatives, xanthine, mercaptans, and other arsenic-containing substances, nucleic acids, nucleoproteins, nucleohistones, etc., among them strongly hygroscopic substances, could be handled without difficulties. Six different techniques for the drying of liquids, three of them for centigram quantities and three for milligram quantities, were tested by application to a group of substances selected so as to represent various types of hygroscopicity, viscosity, and volatility (15). Procedure and apparatus had to be varied depending upon whether or not the drying agent was brought into direct contact with the liquid. Again, if direct contact was chosen, the separation of drying agent and substance could be brought about either by decantation or by distillation. It is expected that the description will permit selection, with considerable assurance of success, of the most suitable procedure for any given liquid.

A simple spring balance which may be used on the work bench and permits weighing to 30 mg. of substance with a precision of ± 0.1 mg. is very convenient in micropreparatory work. An improved model of such a balance is described by Alber (9). The same paper contains the specifications for a porcelain mortar with a conical bowl of approximately 1.5 ml. capacity. About 60 to 90 per cent of 5 to 50 mg. of substance may be recovered from the bowl after grinding.

Apparatus for small-scale catalytic hydrogenation have been described by Prater & Haagen-Smit (16), Johns & Seiferle (17), and Noller & Barusch (18).

Determination of Physical Properties

Concerning general instrumentation the reader is referred to two articles by Müller (19, 20) which in turn give 363 further references to the literature.

Shape, crystallographic constants.—As a rule, reagents are available which produce separation of characteristic well-formed crystals with inorganic ions. Frequently, these reagents are sufficiently selective or specific from a purely chemical viewpoint so that the shape

of the crystals is not actually needed for the purpose of identification but rather serves as a very desirable final confirmation. The situation is, however, quite different in the field of organic analysis. In the majority of tests crystallization is difficult to induce and leads to the separation of skeletal forms or of crystal aggregates rather than to that of well-developed individual crystals. From a chemical viewpoint the reagents are usually selective only for the type of compound but not specific for individual substances. In addition, not many organic compounds give cubic crystals which are easy to identify, and the five crystal systems left, if they can be differentiated under the microscope, are insufficient for the characterization of thousands of substances. It is not surprising under these conditions that it was repeatedly proposed in the earlier stages of the development of microanalysis to use for the identification of compounds the general appearance of a crystal mass on the one hand and the determination of crystallographic constants on the other.

Adelheid Kofler (21) points out, however, that it is very dangerous to draw conclusions on the basis of the general appearance of a crystal mass. The so-called typical forms usually consist of aggregates or skeletons of crystals and are mainly the result of conditions prevailing during the process of separation. Eder & Haas (22), themselves, point out that the process of sublimation must be carefully standardized to allow reproduction of the characteristic forms in the sublimate. Adelheid Kofler points out that the differences in appearance of six sublimates as shown by the photomicrographs of Eder & Haas would not permit identification of any one of the six substances involved. The variability of appearance and the lack of definite distinctive features are further emphasized by Kofler with reference to sublimates of morphine, codeine, thebaine, and papaverine. Three photomicrographs taken of sublimates of each base are definitely different in appearance, and for each of the three different types an exact equivalent can be found among the sublimates obtained with each one of the other three alkaloids. Kofler does not dispute the fact that certain organic substances give crystallizations which can be readily identified. Furthermore, she concedes that the microchemical procedure of precipitation on the microscope slide favors the reproducibility of characteristic forms.

The performance of crystallographic measurements under the polarizing microscope is very simple. Adelheid Kofler points out, however, that the interpretation of the data frequently requires expert

knowledge. Whereas crystals of the cubic and hexagonal classes are relatively easy to identify, the correct interpretation of the forms of the tetragonal, orthorhombic, monoclinic, and triclinic classes requires knowledge of the positions of the crystallographic axes. Only in exceptional instances will the inexperienced be able to arrive at a correct result when attempting a determination of the crystal system by following "simple" directions.

For similar reasons it is difficult to interpret the refractive indices determined on anisotrophic crystals. The determination of the three principal refractive indices of biaxial substances requires knowledge of the orientation of the optic axes. Even the suggestion of Schroeder van der Kolk (*22a*) to determine only the maximum and minimum values for the refractive index has not produced satisfactory results. The refractive indices listed by various authors frequently differ to such an extent as to render them useless for identification purposes. The fact that even expert crystallographers often differ in their findings is in large part explained by failure to differentiate between various states of solvation and between various polymorphic (allotropic) modifications. It is only in recent time that the frequency of the occurrence of polymorphism with organic substances has been properly taken into consideration.

Since it cannot be expected that in the near future all microchemists will have proper crystallographic training and, at the same time, the use of all obtainable data is highly desirable for the identification of organic substances, Kofler suggests that such constants as may be safely identified and reproduced by the less experienced be determined; whether the constants represent characteristic and adequate data for the crystallographer should not be considered. Only well-developed crystals of characteristic shape, which are readily obtained in repeated trial crystallizations, should be chosen for description. An account of the appearance should distinguish between habit (granular, tabulate, prismatic, needle-shaped) and shape. Use of geometrical terms (triangle, square, rectangle, rhombus, rhomboid, pentagon, hexagon, etc., and tetrahedron, cube, octahedron, prism, or double pyramid with 3, 4, 6, 8 sides, etc.) is recommended for the description of the shape. Finally, the following determinations may be performed with little expenditure of time (*23*): measurement of characteristic angles (*24*), detection of birefringency, measurement of extinction angles, determination of refractive index (for both vibration directions with anisotrophic crystals), and determination of

pleochroism. Performance of optical measurements on fragments of anisotrophic crystals should be left to expert crystallographers.

The color of microscopic specimens may be exactly defined with the use of spectroscopic eyepieces (25). The grating microspectrograph of Jelley (26) is easier to manipulate as it is merely necessary to place the microscope under it. Accurate centration is not required, and the microscope is merely moved on the baseboard until the spectrum is uniformly illuminated. Jelley describes use of the instrument in the determination of body color, surface color, and the dispersion of birefringency. The last section of the article gives much valuable information for the study of pleochroic materials.

Accessories of reasonable cost for the conversion of microscopes to polarizing microscopes are described by West (27), and Berkelhamer (28) gives the design of an inexpensive and versatile microscope lamp using fluorescent tube lamps which are known for their low heat output. Chambers, permitting microscopic observation of chemical or biological processes at temperatures from +80 to −180° C. and under pressures varying from vacuum to 75 atm., have been constructed by Harvalik (29). Microradiography as a supplement to microscopy is discussed by Clark & Gross (30).

Density, specific gravity.—A semimicropycnometer of approximately 1 ml. capacity is used by Fontana & Calvin (31) in the determination of the density of heavy water. A vacuum of 0.00001 mm. or better is needed for the filling of the pycnometer. Alber (32) describes pipettes of capacities from 6 c.mm. to 0.1 ml., which are suitable for the determination of the density of organic liquids with a precision of 1 to 10 p.p.th. The determination of the density of solids was carried out with 0.05 to 0.25 mg. of material by means of the flotation method of Bernal & Crowfoot (32*a*). The densities of the liquids in which the crystals remained suspended were determined with the use of the pipettes. The falling-drop method was applied by Barbour (33) to the determination of serum albumin in 1 ml. samples. A modified micropipette for use in the falling-drop method has been described by Rosebury & van Heyningen (34). Mortensen (35) designed a pipette containing two hollow glass beads, one slightly lighter and the other slightly heavier than the specific gravity range of the serums examined, for the rapid determination of serum protein in 1 ml. fluid.

Refractive index.—Because of the difficulties associated with the interpretation of the refractive indices of anisotrophic solids, L. Kofler

(36) applies the immersion method to the melt of organic substances obtained in the determination of the melting point under the microscope. When monochromatic light is used, the outlines of the glass fragments used as standards of reference are made to disappear completely by proper adjustment of the temperature. A series of twenty-three glass powders (n from 1.3400 to 1.6718) is now available (37, 38). Reimers (39) applied the method to the identification of related substances, and he found that identity of melting points and refractive indices does not occur with exceptional frequency for isomers or other closely related compounds. A new attachment to the Fisher refractometer permits determination of the melting point (up to 200° C., accuracy of 1° to 2° C.) and of the refractive index (range 1.300 to 1.900, accuracy ± 0.002) of the melt of a few milligrams of substance (40). The dispersion may be estimated. The instrument contains a glass trough which holds the "liquid prism" of the substance. Simultaneous observation of the relative positions of an illuminated slit and its virtual image, seen through the liquid or melt, permits the reading of the refractive index units directly off a scale.

The microrefractometer of Edwards & Otto (41) is based on the same principle as the Fisher instrument. It may be made at small cost and allows determination of the refractive index of liquids with an accuracy of ± 0.003. Approximately 10 c.mm. of substance are required, and a suitable heating unit can be attached. Kirk & Gibson (42) and Alber & Bryant (43) describe apparatus suited to the determination of the refractive index of volatile and viscous liquids. An accuracy of ± 0.0005 to ± 0.002 is obtained, and only 5 to 8 mg. of substance are required. Kirk & Gibson avoid the use of a cement by drilling a well of 1 mm. diameter and 5 mm. depth into a glass plate. This well serves as cell for the liquid, and the refractive index is determined by the method of the Duc de Chaulnes (25) which requires a microscope with graduated micrometer drum for fine adjustment. Alber & Bryant use the cell of Nichols (43a), which is formed by a metal ring on a slide and contains two small glass prisms. With two such cells the range from 1.40 to 2.00 may be covered. Because of the use of cement, liquids of marked solvent action must not be left longer in the cells than necessary. More than 50 per cent of the sample may be recovered and used for further tests.

The ultramicroscopic refractometer of von Engelhardt (44) permits estimation of the refractive indices of particles of less than 1 μ. radius.

Melting point, transition points.—Hot stages for the determination of transition points under the microscope have been known for a long time (25). Nevertheless, their use did not become popular, and the explanation is obviously furnished by the multiplicity of phenomena which is frequently observed with the microscope and which must be confusing to an occasional user of the hot stage. In addition, and without fault on the part of the apparatus, the melting temperatures observed under the microscope are often not in agreement with those determined with the melting point capillary and listed in the literature. Thus, the systematic investigation of Kofler *et al.* (21, 45, 46, 47, 48) was necessary to clarify the relation between macro and micro melting points and to collect the data required for use of the micromethod in the identification of organic compounds. Of necessity this work led among others to the discovery of heretofore unsuspected allotropic modifications of various familiar organic substances.

The distinctive feature of the determination of the melting point under the microscope is the fact that individual particles which are not in contact with one another are observed in the microscopic field. If the sample contains particles representing different states of solvation or different polymorphic modifications, divers types of shape may immediately be noticed on the slide, and on heating each particle will behave according to its nature. One may be able to observe the melting points of hydrate and anhydrous compound or of different allotropic forms on the various specimens visible in the microscopic field. The material may be uniform at the start, and some particles may undergo dehydration or transition to other allotropic modifications while others remain unchanged and give the melting points of the hydrate or modification present at the outset. Transition from one state to the other may be merely indicated by a change in the transparency of the particle. In other instances, particles melt and crystals of a different type separate from the drop. Sublimation may occur, and characteristic changes and melting points may be observed on the sublimate. Obviously, determination of the melting point under the microscope is able to furnish more information than the customary capillary method.

Rimington & Symons (49) used the microscope hot stage of Kofler in the determination of the melting points of porphyrins and found that little change could be detected in the microscopic appearance when liquefaction of the dark red particles occurred. The exact

moment of melting was then ascertained with the use of crossed nicols. Zscheile & White (50) constructed for the investigation of carotenoid pigments a microscope hot stage with which it is possible to determine the melting points of single crystals as a sharply defined constant (precision ±0.002° to 0.04° C.; range from room temperature to 250° C.). The material under investigation is surrounded with nitrogen, and the melting point of anisotropic crystals is determined by observation of the sharp change in the transmission of polarized light.

Surface tension, film pressure.—Boyd & Harkins (51) discuss the film balance as an analytical tool for biological and food research. Description of apparatus and technique includes the determination of the viscosity of monolayers.

Optical rotation.—Marion (52) describes the adaptation of the polarizing microscope for use as a polarimeter. The specimen tube of 5 cm. length has a capacity of 0.15 ml. Sugar solutions were made up in a 1 ml. volumetric flask and used for tests. Calculated and observed angles of rotation agreed within ±35 p.p.th.

Electrophoresis.—A cell for the measurement of migration velocities of suspended particles is described by Briggs (53). It is of simple design and may be prepared with relative ease.

Qualitative Analysis

Since purely qualitative findings are of doubtful value, quantitative aspects have been increasingly emphasized in recent years. It is now time to point out that qualitative procedures permitting semiquantitative estimations (54) may be employed to advantage in place of quantitative methods whenever a high precision is not needed. In addition, in qualitative analysis, the substances in question are usually isolated in a form which can be preserved for further reference, whereas the use of colorimetric and titrimetric procedures of quantitative analysis usually leads to the destruction of evidence.

Apparatus for general use.—A practical kit for reagents and apparatus has been described by Cool (55). Heating blocks for a variety of uses have been designed by Morton & Mahoney (56) and by Noller (57). Kurtz (58) found it possible to evaporate 0.3 ml. of an aqueous solution within two or three minutes by placing the centrifuge cone into a steam jacket and blowing a jet of electrically heated air on the surface of the solution in the cone. As the solution does not boil, losses due to spattering need not be feared. Infrared lamps may be

used for heating solutions on a slide or tests on a spot plate (59). Apparatus for filtration on a centigram scale is described by Barber (60). Suction and pressure are supplied by the standard ear and ulcer syringe No. 527, 60 ml. capacity, of the Davol Rubber Company, Providence, R.I. The technique may be useful in field work when neither centrifuge nor suction pump is available.

Microgram technique of qualitative analysis.—A technique for chemical work on 1 to 0.001 μg. of material has been applied to the qualitative analysis of inorganic materials (3, 61). The work is performed in capillary cones, i.e., small centrifuge cones of 2 c.mm. capacity. Most manipulations are performed under the low power microscope with the aid of a simple manipulator. A micropipette with somewhat wider tip than have those which are used in microbiological work serves in the measuring and transferring of liquids. The operations of any standard scheme of inorganic qualitative analysis, including distillation, may be performed with confidence of success, and the quantities of the isolated constituents can be estimated.

Electrolytic methods.—Yagoda (62) applies the electrographic method to the investigation of polished specimens for the localization of chloride, sodium, and potassium in sections of plant and animal tissues. Cuts of approximately 3 mm. thickness are sandwiched together with a matrix (gelatin-coated paper, plane porous casting of plaster of Paris) and briefly electrolyzed between two aluminum electrodes. The print is then developed in the matrix, and it is capable of rendering sharply defined patterns in terms of specific ions.

The polarographic method is now well established for the detection and estimation of inorganic and organic substances. Summarizing accounts have been recently presented by Kolthoff & Lingane (63, 64) and by Müller (65, 66). Furman, Bricker & Whitesell (67) write on the construction and operation of the polarograph, and Lingane (68) reports a procedure for simplified calibration of the mercury electrode. Recent papers deal with the determination of arsenic (69), of nitrate in blood and urine (70), the study of quinones related to vitamin E (71), and the amperometric titration of α-tocopherol (72).

Organic substances.—The qualitative microanalysis of organic substances has undergone changes which promise increased usefulness. Three outstanding trends may be easily recognized:

(*a*) Systematic development of procedures for the isolation and purification of compounds previous to determination of physical con-

stants and ultimate analysis. This line has been followed by Alber *et al.* (9, 12, 14, 15), and the procedures were calculated to take care of decigrams, centigrams, or at least several milligrams of material so as to have sufficient substance for the performance of ultimate analysis. Methods for the determination of physical constants are designed with special attention to quantitative recovery of material (32, 43). This approach is especially adapted to the investigation of new compounds.

(*b*) Application of comprehensive schemes of organic qualitative analysis (Mulliken & Huntress; Kamm, Shriner & Fuson) to the identification of small quantities of known compounds. It may be expected that existing procedures will serve in the separation. The classification of milligram to centigram quantities of organic compounds by means of physical and chemical tests has been extensively investigated (73 to 78). Schneider *et al.* discuss preliminary examination by ignition test (74); test for nitrogen, sulfur, and halogens (74); classification reactions of compounds of carbon, hydrogen, and oxygen (76, 77); and identification of compounds containing nitrogen (78). Two papers (74, 75) deal with the classification according to solubility. The determination is performed in a capillary if semiquantitative results are desired. For very rapid determination of the degree of solubility, the sensitivity of the *schlieren* test of Emich (79) was intentionally reduced.

(*c*) Identification of very small quantities (less than 1 mg. to 1 μg.) of organic substances. Kofler recommends determination of the melting point under the microscope (21), followed by the determination of the refractive index of the melt (36). The shape of well-developed crystals and safely reproducible crystallographic data (21) are suggested as additional aids in confirmation or differentiation. Of course, only substances which are already known may be recognized or identified in this manner. Accumulation of the required data has been aided recently by investigations on the esters of carbanilic acid with various alcohols (80, 81), the 2,4-dinitro-phenylhydrazones of various aldehydes and ketones (82), on sugars and polyhydric alcohols (83), on the osazones of sugars (84), the silver salts of amino acids (85), and on derivatives of amytal, pentobarbital, and dial (86).

The classical method of separation and identification by tests on the microscope slide has been applied by Whitmore *et al.* to substituted naphthalenesulfonic acids (87) and to alkaloids (88, 89). Further slide tests for alkaloids have been described by Berisso (90),

Fulton (91), Malowan (92), Martini (93, 94), and White (95). A slide test for vitamin B_1 is reported by da Silva Pereira (96).

Chromatography.—Comprehensive information may be found in the book of Zechmeister & Cholnoky (97) and in the review by Strain (98). A microtechnique of chromatographic analysis has been described by Crowe (99). The chromatographic separation of mixtures of amino acids is discussed by Wachtel & Cassidy (100). Erlenmeyer & Schmidlin (101) deal with the separation of potassium and sodium.

Quantitative Analysis

Balances, gravimetric determinations.—Determination of the precision of a single weighing (102) has shown that many analytical balances may be used for microwork. The minimum size of the sample may be calculated as a function of the weighing error, the composition of the weighing form, and the approximate composition of the material to be analyzed (103). The practical applicability of the derived simple relations has been demonstrated by the investigations of Niederl, Niederl & Nagel (104), of Paulson (105), and by the more involved analyses performed by Llacer & Sozzi (106, 107). The work of Conrady (108) indicates that the performance of a weighing produces a temporary bias in the indication of balances because of deformation of the knife edge. This effect does not seem to be strong enough, at least with small loads, to completely invalidate the customary procedure for the determination of the precision of balances (3, 102). The paper of Vernon (109) contains much valuable information on selection of analytical balances for precision work and on auxiliary devices and technique designed to improve the precision of the instrument. Of course, the double-transposition procedure of Conrady would be too cumbersome for the purpose of analysts. Vollrath's (110) electromagnetic device for starting the swinging of a balance should be mentioned in this connection.

Only recently the precision of microchemical balances has been studied by a rigorous statistical treatment. The Divisional Committee on Microbalances of the American Chemical Society reports (111) a probable error of between 2 and 3 μg. for the individual weighing. This figure represents an estimated average derived from weighings with 1 and 10 gm. loads on twenty-two different balances. Corner & Hunter (112) arrive at an estimate of ±3 μg. from a study of seven balances. The major portion of the uncertainty, ±2.4 μg.,

is caused by the difficulty in reproducing the exact position of the rider, a problem which has been studied in great detail by Ramberg (cf 113). The effect of a temperature differential between object and balance has been investigated by Blade (114). A fuzz detector is described by Hayman & Reiss (115). Van Straten & Ehret (116) and Rodden (117) give directions for the removal of static charges from glassware. A desiccator in which a dry atmosphere is continuously maintained is described by Ramberg (118).

A spring balance giving a precision of ± 0.1 μg. with a maximum load of 0.3 mg. is used by Lowry (119). A fine quartz capillary of 20 cm. length with a small v-shaped bend at the free end is mounted in a 1-gallon tin can. The displacement of the tip of the capillary is read with a cathetometer to 0.01 mm. Since the apparatus has to be extremely light because of the small capacity of the balance, loops of 2 mm. diameter are used to hold the material under investigation. Loops of this dimension are formed at both ends of fine quartz fibers of 1 cm. length; of course, one loop is needed for suspending the device from the hook at the end of the spring. The balance is calibrated by transferring measured volumes of salt solutions of known concentration to a loop and weighing the residue after complete evaporation of the solvent. Residue determinations were performed with samples of 10 to 100 μg. mass, and the distribution of dry matter and fat was measured in hog gastric mucosa. A comprehensive and up to date treatise of the field of gravimetric microanalysis is given by Hecht & Donau (120).

Titrimetric analysis.—Burettes which use surface tension effects in place of a stopcock are able to deliver at any slow rate desired. They may be extremely simple in design, and they are very easy to operate. The model used by Hybbinette (3, 121) consists of a horizontal tube backed by a millimeter scale, which is bent at one end to form a perpendicular tip. The practically identical burette of Hawes & Skavinski (122) has a leveling device for the regulation of the rate of outflow. This feature may be practical for routine work, but it is actually not desirable for the attainment of high precision. If the calibrated tube of microburettes operated by surface tension effects is in a perpendicular position, it becomes necessary to regulate the gas pressure above the standard solution. This is usually accomplishd by means of some mercury leveling device as used in the burettes of Linderstrøm-Lang & Holter (122a) and of Heatley (123). If, however, the gas space of the burette is flexibly connected

to a large reservoir in which the pressure can be varied at will in a controllable manner, the burette then may be operated by remote control in any position and in a sealed space, if so desired. A burette of this type has been tested by Llacer & Sozzi (3).

Of the burettes of the syringe type (124) the one described by Ogg, Johns, Hoecker & Hammer (125) consists of a graduated tube with a fine tip, a short piece of rubber tubing, and a screw clamp. Scholander (126) as well as Dean & Fetcher (127) advance the plunger by means of a caliper.

The evaporation of standard solution from the tips of microburettes of small capacity may introduce considerable errors which, however, may be avoided by simple precautions (128). Practice and theoretical foundations of titrimetry with milligram samples are extensively treated by Mika (129). The author agrees with the generally accepted compromise to apply relatively large corrections for the blank rather than to maintain the concentrations of standard solutions as employed in macrotitrations and to work with essentially smaller volumes of solutions than were customary up to this time in microtitrations. Ways of maintaining the standard conditions of macrotitrimetry are discussed in connection with the experimental work of Siggia (130). Retention of standard conditions in microgram procedures leads to inconveniently small volumes of solutions (3), and whereas construction of a suitable burette is relatively simple, great difficulties are met in designing a titration cell which permits efficient stirring and recognition of the end point.

Volumetric flasks of 1 to 10 ml. capacity have been described by Caley (131) and Holmes (132), and the silver reductor has been used by Edmonds & Birnbaum (133) on a milligram scale. Concerning the general advancements in titrimetry the reader is referred to the recent reviews of Furman (134) on potentiometric titrations and Kolthoff & Cohn (135) on amperometric titrations.

Organic elementary analysis.—A collection of the literature up to and including 1941 may be found in the book by Niederl & Niederl (136). Attention is called to the isothermic method for the determination of molecular weight (pp. 230 to 238), for its application to substances of high molecular weight seems possible. Very recent publications deal with the method of Dumas (137, 138), the method of Kjeldahl (139 to 143), the diffusion method (122, 144, 145, 146), the semimicrodetermination of carbon (147, 148), the determination of sulfur (149, 150), determination of bromine and iodine (151), of

phosphorus in lipid extracts (152), and of arsenic (153). MacNevin & Brown (151) test the bomb of their lime fusion method for tightness of seal by half filling it with dry ice, sealing, and inverting in a beaker of warm water. Brewster & Rieman (149) burn compounds containing sulfur, nitrogen, and halogen in the spiral tube. The rinsings are evaporated on the steam bath, and the sulfuric acid remaining in the residue is titrated with standard alkali. Correction for the blank is required, but the results listed are excellent. Samsel & McHard (154) studied the determination of alkoxyl groups on 50 to 60 mg. samples of cellulose ethers.

Volumetric analysis of gases.—Van Slyke & Kreysa (155) determine the calcium in 0.2 ml. of serum by precipitation as picrolonate, wet combustion of the precipitate, and manometric measurement of the liberated carbon dioxide. Bunzell (156) describes a relatively simple and rapid method which permits estimation of 0.01 μg. of vitamin B_1 with fair accuracy. It is based on stimulation of yeast growth and manometric measurement of the carbon dioxide evolved. A procedure for the estimation of thiamin using the Warburg technique and applicable to tissues and fluids low in thiamin (0.01 μg. per ml.) is described by Josephson & Harris (157).

Gas analysis.—New combustion coils for the burning of gases are described by Blacet, Sellers & Blaedel (158) and by Smith & Leighton (159). The former also describe a change in the procedure of preparing a cupric oxide-potassium hydroxide bead for the determination of hydrogen. The latter determine nitric oxide in mixtures with hydrogen and nitrogen by addition of oxygen in the presence of potassium hydroxide and analysis of the remaining gas. Ammonia is separated from nitric oxide by absorption on a bead of monochloroacetic acid. Scholander (160, 161, 162) describes a modification of the gas burette of Krogh. The plunger is advanced into the mercury by means of a caliper, and the displacement is read on the micrometer drum. The upper meniscus of the gas column is always returned to the same mark before a reading is made. A surface-active agent is added to the aqueous solutions (163, 164). Carbon dioxide and oxygen in 1 ml. to 10 c.mm. of respiratory gas may be determined within six to ten minutes with a precision of ± 0.02 to ± 0.1 per cent (0.01 c.mm. per hour). Gilbert & Tannich (165) report on a reasonably simple method for the estimation and detection of aromatic vapors in the atmosphere, which gives an accuracy of 15 p.p.m.

This report is confined to a few selected topics. Additional information may be gained from a comprehensive and profusely illustrated review of organic microchemistry by Hallett (166).

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